EXHIBIT 4

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April 10, 2001

Patricia Granahan, Esq.
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Cambridge, Massachusetts 02142

Subject: U.S. Filing Date: March 30, 2001
Title: RNA Sequence-Specific Mediators of RNA Interference
Inventors: Thomas Tuschl, Phillip D. Zamore, Phillip A. Sharp and David P. Bartel
Our Docket No.: 0399.2008-002
Your Reference No.: WHI00-06; MIT-8768W

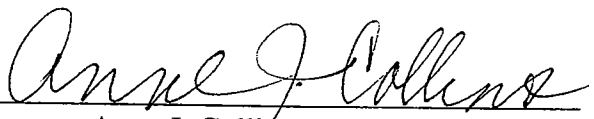
Dear Pat:

Enclosed is a copy of the referenced application which was filed via the Express Mail Procedure in the U.S. Patent and Trademark Office on March 30, 2001.

If you have any questions, please feel free to contact me.

Very truly yours,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

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HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

UTILITY PATENT APPLICATION TRANSMITTAL (Only for new nonprovisional applications under 37 C.F.R. 1.53(b))	Attorney Docket No.	0399.2008-002
	First Named Inventor or Application Identifier	Thomas Tuschl
	Express Mail Label No.	EL762342636US

Title of Invention: RNA SEQUENCE-SPECIFIC MEDIATORS OF RNA INTERFERENCE

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: **Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231**

1. [] Fee Transmittal Form *(Submit an original, and a duplicate for fee processing)*
2. [X] Specification **[Total Pages 60]** *(preferred arrangement set forth below)*
 - Descriptive title of the invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to microfiche Appendix
 - Background of the Invention
 - Summary of the Invention
 - Brief Description of the Drawings
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. [X] Drawing(s) (35 U.S.C. 113) **[Total Sheets 11]**
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4. [] Oath or Declaration/POA **[Total Pages []]**
 a. [] Newly executed (original or copy)
 b. [] Copy from a prior application (37 C.F.R. 1.63(d)) *(for continuation/divisional with Box 18 completed)* **[NOTE Box 5 below]**
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5. [] Incorporation By Reference *(useable if Box 4b is checked)* The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. [] Microfiche Computer Program *(Appendix)*
7. [] Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
 a. [] Computer Readable Copy
 b. [] Paper Copy (identical to computer copy) **[] Pages**
 c. [] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. [] Assignment Papers (cover sheet & documents)
 [X] Assignees - Whitehead Institute for Biomedical Research, Cambridge, MA; Max-Planck-Gesellschaft zur Förderung der Wissenschaften E.V., Munich, Germany; Massachusetts Institute of Technology, Cambridge, MA; and University of Massachusetts Medical Center, Worcester, MA.
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 Prior application information: Examiner: Group Art Unit:

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Attorney's Docket No.: 0399.2008-002

RNA Sequence-Specific Mediators of RNA Interference

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/265,232, filed January 31, 2001 and U.S. Provisional Application No. 60/193,594, filed March 30, 2000, and claims priority under 35 U.S.C. §119 to European Application No. 00 126 325.0 filed December 1, 2000. The entire teachings of the above applications are incorporated herein by reference.

GOVERNMENT SUPPORT

Work described herein was funded in part by grants from the National Institutes of Health through a United States Public Health Service MERIT award (Grant No. RO1-GM34277) from the National Institutes of Health. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (Fire et al. (1998) Nature 391, 806-811). dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. RNAi involves mRNA degradation, but many of the biochemical mechanisms underlying this

interference are unknown. The recapitulation of the essential features of RNAi in vitro is needed for a biochemical analysis of the phenomenon.

SUMMARY OF THE INVENTION

Described herein is gene-specific, dsRNA-mediated interference in a cell-free system derived from syncytial blastoderm Drosophila embryos. The in vitro system complements genetic approaches to dissecting the molecular basis of RNAi. As described herein, the molecular mechanisms underlying RNAi were examined using the Drosophila in vitro system. Results showed that RNAi is ATP-dependent yet uncoupled from mRNA translation. That is, protein synthesis is not required for RNAi in vitro. In the RNAi reaction, both strands (sense and antisense) of the dsRNA are processed to small RNA fragments or segments of from about 21 to about 23 nucleotides (nt) in length (RNAs with mobility in sequencing gels that correspond to markers that are 21-23 nt in length, optionally referred to as 21-23 nt RNA). Processing of the dsRNA to the small RNA fragments does not require the targeted mRNA, which demonstrates that the small RNA species is generated by processing of the dsRNA and not as a product of dsRNA-targeted mRNA degradation. The mRNA is cleaved only within the region of identity with the dsRNA. Cleavage occurs at sites 21-23 nucleotides apart, the same interval observed for the dsRNA itself, suggesting that the 21-23 nucleotide fragments from the dsRNA are guiding mRNA cleavage. That purified 21-23 nt RNAs mediate RNAi confirms that these fragments are guiding mRNA cleavage.

Accordingly, the present invention relates to isolated RNA molecules (double-stranded; single-stranded) of from about 21 to about 23 nucleotides which mediate RNAi. That is, the isolated RNAs of the present invention mediate degradation of mRNA of a gene to which the mRNA corresponds (mediate degradation of mRNA that is the transcriptional product of the gene, which is also referred to as a target gene). For convenience, such mRNA is also referred to herein as mRNA to be degraded. As used herein, the terms RNA, RNA molecule(s), RNA segment(s) and RNA fragment(s) are used interchangeably to refer to RNA that mediates RNA interference. These terms

include double-stranded RNA, single-stranded RNA, isolated RNA (partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA), as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the 21-23 nt RNA or internally (at one or more nucleotides of the RNA). Nucleotides in the RNA molecules of the present invention can also comprise non-standard nucleotides, including non-naturally occurring nucleotides or deoxyribonucleotides. Collectively, all such altered RNAs are referred to as analogs or analogs of naturally-occurring RNA. RNA of 21-23 nucleotides of the present invention need only be sufficiently similar to natural RNA that it has the ability to mediate (mediates) RNAi. As used herein the phrase "mediates RNAi" refers to (indicates) the ability to distinguish which RNAs are to be degraded by the RNAi machinery or process. RNA that mediates RNAi interacts with the RNAi machinery such that it directs the machinery to degrade particular mRNAs. In one embodiment, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that direct cleavage of specific mRNA to which their sequence corresponds. It is not necessary that there be perfect correspondence of the sequences, but the correspondence must be sufficient to enable the RNA to direct RNAi cleavage of the target mRNA. In a particular embodiment, the 21-23 nt RNA molecules of the present invention comprise a 3' hydroxyl group.

The present invention also relates to methods of producing RNA molecules of about 21 to about 23 nucleotides with the ability to mediate RNAi cleavage. In one embodiment, the Drosophila in vitro system is used. In this embodiment, dsRNA is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA molecules of about 21 to about 23 nucleotides. In another embodiment, the Drosophila in vitro system is used to obtain RNA sequences of about 21 to about 23 nucleotides which mediate RNA interference of the mRNA of a particular gene (e.g., oncogene, viral gene). In this embodiment, double-stranded RNA

that corresponds to a sequence of the gene to be targeted is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides in length. As shown herein, 21-23 nt RNA mediates RNAi of the mRNA of the targeted gene (the gene whose mRNA is to be degraded). The method of obtaining 21-23 nt RNAs using the Drosophila in vitro system can further comprise isolating the RNA sequence from the combination.

The present invention also relates to 21-23 nt RNA produced by the methods of the present invention, as well as to 21-23 nt RNAs, produced by other methods, such as chemical synthesis or recombinant DNA techniques, that have the same or substantially the same sequences as naturally-occurring RNAs that mediate RNAi, such as those produced by the methods of the present invention. All of these are referred to as 21-23 nt RNAs that mediate RNA interference. As used herein, the term isolated RNA includes RNA obtained by any means, including processing or cleavage of dsRNA as described herein; production by chemical synthetic methods; and production by recombinant DNA techniques. The invention further relates to uses of the 21-23 nt RNAs, such as for therapeutic or prophylactic treatment and compositions comprising 21-23 nt RNAs that mediate RNAi, such as pharmaceutical compositions comprising 21-23 nt RNAs and an appropriate carrier (e.g., a buffer or water).

The present invention also relates to a method of mediating RNA interference of mRNA of a gene in a cell or organism (e.g., mammal such as a mouse or a human). In one embodiment, RNA of about 21 to about 23 nt which targets the mRNA to be degraded is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of the mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism. The cell or organism can be one in which RNAi occurs as the cell or organism is obtained or a cell or organism can be one that has been modified so that RNAi occurs (e.g., by addition of components obtained from a cell or cell extract that mediate RNAi or activation of endogenous components). As used herein, the term "cell or organism in which RNAi

occurs" includes both a cell or organism in which RNAi occurs as the cell or organism is obtained, or a cell or organism that has been modified so that RNAi occurs. In another embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNAs of about 21 to about 23 nucleotides. 21 to 23 nt RNA is then isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the gene in the cell or organism. As described for the previous embodiment, the cell or organism is one in which RNAi occurs naturally (in the cell or organism as obtained) or has been modified in such a manner that RNAi occurs. 21 to 23 nt RNAs can also be produced by other methods, such as chemical synthetic methods or recombinant DNA techniques.

The present invention also relates to biochemical components of a cell, such as a Drosophila cell, that process dsRNA to RNA of about 21 to about 23 nucleotides. In addition, biochemical components of a cell that are involved in targeting of mRNA by RNA of about 21 to about 23 nucleotides are the subject of the present invention. In both embodiments, the biochemical components can be obtained from a cell in which they occur or can be produced by other methods, such as chemical synthesis or recombinant DNA methods. As used herein, the term "isolated" includes materials (e.g., biochemical components, RNA) obtained from a source in which they occur and materials produced by methods such as chemical synthesis or recombinant nucleic acid (DNA, RNA) methods.

The present invention also relates to a method for knocking down (partially or completely) the targeted gene, thus providing an alternative to presently available methods of knocking down (or out) a gene or genes. This method of knocking down gene expression can be used therapeutically or for research (e.g., to generate models of disease states, to examine the function of a gene, to assess whether an agent acts on a

gene, to validate targets for drug discovery). In those instances in which gene function is eliminated, the resulting cell or organism can also be referred to as a knockout. One embodiment of the method of producing knockdown cells and organisms comprises introducing into a cell or organism in which a gene (referred to as a targeted gene) is to be knocked down, RNA of about 21 to about 23 nt that targets the gene and maintaining the resulting cell or organism under conditions under which RNAi occurs, resulting in degradation of the mRNA of the targeted gene, thereby producing knockdown cells or organisms. Knockdown cells and organisms produced by the present method are also the subject of this invention.

The present invention also relates to a method of examining or assessing the function of a gene in a cell or organism. In one embodiment, RNA of about 21 to about 23 nt which targets mRNA of the gene for degradation is introduced into a cell or organism in which RNAi occurs. The cell or organism is referred to as a test cell or organism. The test cell or organism is maintained under conditions under which degradation of mRNA of the gene occurs. The phenotype of the test cell or organism is then observed and compared to that of an appropriate control cell or organism, such as a corresponding cell or organism that is treated in the same manner except that the targeted (specific) gene is not targeted. A 21 to 23 nt RNA that does not target the mRNA for degradation can be introduced into the control cell or organism in place of the RNA introduced into the test cell or organism, although it is not necessary to do so. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the degraded mRNA. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract that mediates RNAi, such as the soluble extract derived from Drosophila embryo described herein, under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into a cell or organism in which RNAi occurs (test cell or test organism). The test cell or test organism is maintained under conditions under which degradation of the mRNA occurs. The phenotype of the

test cell or organism is then observed and compared to that of an appropriate control, such as a corresponding cell or organism that is treated in the same manner as the test cell or organism except that the targeted gene is not targeted. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the targeted gene. The information provided may be sufficient to identify (define) the function of the gene or may be used in conjunction with information obtained from other assays or analyses to do so.

Also the subject of the present invention is a method of validating whether an agent acts on a gene. In this method, RNA of from about 21 to about 23 nucleotides that targets the mRNA to be degraded is introduced into a cell or organism in which RNAi occurs. The cell or organism (which contains the introduced RNA) is maintained under conditions under which degradation of mRNA occurs, and the agent is introduced into the cell or organism. Whether the agent has an effect on the cell or organism is determined; if the agent has no effect on the cell or organism, then the agent acts on the gene.

The present invention also relates to a method of validating whether a gene product is a target for drug discovery or development. RNA of from about 21 to about 23 nucleotides that targets the mRNA that corresponds to the gene for degradation is introduced into a cell or organism. The cell or organism is maintained under conditions in which degradation of the mRNA occurs, resulting in decreased expression of the gene. Whether decreased expression of the gene has an effect on the cell or organism is determined, wherein if decreased expression of the gene has an effect, then the gene product is a target for drug discovery or development.

The present invention also encompasses a method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNA of from about 21 to about 23 nucleotides which targets the mRNA of the protein (the mRNA that encodes the protein) for degradation. As a result, the protein is not produced or is not produced to the extent it would be in the absence of the treatment.

Also encompassed by the present invention is a gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

Also encompassed by the present invention is a method of identifying target sites within an mRNA that are particularly suitable for RNAi as well as a method of assessing the ability of 21-23 nt RNAs to mediate RNAi.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1 is a schematic representation of reporter mRNAs and dsRNAs Rr-Luc and Pp-Luc. Lengths and positions of the ssRNA, asRNA, and dsRNAs are shown as black bars relative to the Rr-Luc and Pp-Luc reporter mRNA sequences. Black rectangles indicate the two unrelated luciferase coding sequences, lines correspond to the 5' and 3' untranslated regions of the mRNAs.

Figure 2A is a graph of the ratio of luciferase activities after targeting 50 pM Pp-Luc mRNA with 10 nM ssRNA, asRNA, or dsRNA from the 505 bp segment of the Pp-Luc gene showing gene-specific interference by dsRNA in vitro. The data are the average values of seven trials ± standard deviation. Four independently prepared lysates were used. Luciferase activity was normalized to the buffer control; a ratio equal to one indicates no gene-specific interference.

Figure 2B is a graph of the ratio of luciferase activities after targeting 50 pM Rr-Luc mRNA with 10 nM ssRNA, asRNA, or dsRNA from the 501 bp segment of the Rr-Luc gene showing gene-specific interference by dsRNA in vitro. The data are the average values of six trials ± standard deviation. A Rr-Luc/Pp-Luc ratio equal to one indicates no gene-specific interference.

Figure 3A is a schematic representation of the experimental strategy used to show that incubation in the Drosophila embryo lysate potentiates dsRNA for gene-

specific interference. The same dsRNAs used in Figure 2 (or buffer) was serially preincubated using two-fold dilutions in six successive reactions with Drosophila embryo lysate, then tested for its capacity to block mRNA expression. As a control, the same amount of dsRNA (10 nM) or buffer was diluted directly in buffer and incubated with Pp-Luc and Rr-Luc mRNAs and lysate.

Figure 3B is a graph of potentiation when targeting Pp-Luc mRNA. Black columns indicate the dsRNA or the buffer was serially preincubated; white columns correspond to a direct 32-fold dilution of the dsRNA. Values were normalized to those of the buffer controls.

Figure 3C is a graph of potentiation when targeting Rr-Luc mRNA. The corresponding buffer control is shown in Figure 3B.

Figure 4 is a graph showing effect of competitor dsRNA on gene-specific interference. Increasing concentrations of nanos dsRNA (508 bp) were added to reactions containing 5 nM dsRNA (the same dsRNAs used in Figures 2A and 2B) targeting Pp-Luc mRNA (black columns, left axis) or Rr-Luc mRNA (white columns, right axis). Each reaction contained both a target mRNA (Pp-Luc for the black columns, Rr-Luc for the white) and an unrelated control mRNA (Rr-Luc for the black columns, Pp-Luc for the white). Values were normalized to the buffer control (not shown). The reactions were incubated under standard conditions (see Methods).

Figure 5A is a graph showing the effect of dsRNA on mRNA stability. Circles, Pp-Luc mRNA; squares, Rr-Luc mRNA; filled symbols, buffer incubation; open symbols, incubation with Pp-dsRNA.

Figure 5B is a graph showing the stability of Rr-Luc mRNA incubated with Rr-dsRNA or Pp-dsRNA. Filled squares, buffer; open squares, Pp-dsRNA (10 nM); open circles, Rr-dsRNA (10 nM).

Figure 5C is a graph showing the dependence on dsRNA length. The stability of the Pp-Luc mRNA was assessed after incubation in lysate in the presence of buffer or dsRNAs of different lengths. Filled squares, buffer; open circles, 49 bp dsRNA (10 nM); open inverted triangles, 149 bp dsRNA (10 nM); open triangles, 505 bp dsRNA

(10 nM); open diamonds, 997 bp dsRNA (10 nM). Reactions were incubated under standard conditions (see Methods).

Figure 6 is a graph showing that RNAi Requires ATP. Creatine kinase (CK) uses creatine phosphate (CP) to regenerate ATP. Circles, +ATP, +CP, +CK; squares, -ATP, +CP, +CK; triangles, -ATP, -CP, +CK; inverted triangles, -ATP, +CP, -CK.

Figure 7A is a graph of protein synthesis, as reflected by luciferase activity produced after incubation of Rr-luc mRNA in the in vitro RNAi reaction for 1 hour, in the presence of the protein synthesis inhibitors anisomycin, cycloheximide, or chloramphenicol, relative to a reaction without any inhibitor showing that RNAi does not require mRNA translation.

Figure 7B is a graph showing translation of 7-methyl-guanosine- and adenosine-capped Pp-luc mRNAs (circles and squares, respectively) in the RNAi reaction in the absence of dsRNA, as measured by luciferase activity produced in a one-hour incubation.

Figure 7C is a graph showing incubation in an RNAi reaction of uniformly ^{32}P-radiolabeled 7-methyl-guanosine-capped Pp-luc mRNA (circles) and adenosine-capped Pp-luc mRNA (squares), in the presence (open symbols) and absence (filled symbols) of 505 bp Pp-luc dsRNA.

Figure 8A is a graph of the of the denaturing agarose-gel analysis of Pp-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt Pp-asRNA, or 505 bp Pp-dsRNA for the times indicated showing that asRNA causes a small amount of RNAi in vitro.

Figure 8B is a graph of the of the denaturing agarose-gel analysis of Rr-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt Pp-asRNA, or 505 bp Pp-dsRNA for the times indicated showing that asRNA causes a small amount of RNAi in vitro.

Figure 9 is a schematic of the positions of the three dsRNAs, 'A,' 'B,' and 'C,' relative to the Rr-luc mRNA.

Figure10 indicates the cleavage sites mapped onto the first 267 nt of the Rr-luc mRNA (SEQ ID NO: 1). The blue bar below the sequence indicates the position of dsRNA 'C,' and blue circles indicate the position of cleavage sites caused by this dsRNA. The green bar denotes the position of dsRNA 'B,' and green circles, the cleavage sites. The magenta bar indicates the position of dsRNA 'A,' and magenta circles, the cleavages. An exceptional cleavage within a run of 7 uracils is marked with a red arrowhead.

Figure 11 is a proposed model for RNAi. RNAi is envisioned to begin with cleavage of the dsRNA to 21-23 nt products by a dsRNA-specific nuclease, perhaps in a multiprotein complex. These short dsRNAs might then be dissociated by an ATP-dependent helicase, possibly a component of the initial complex, to 21-23 nt asRNAs that could then target the mRNA for cleavage. The short asRNAs are imagined to remain associated with the RNAi-specific proteins (circles) that were originally bound by the full-length dsRNA, thus explaining the inefficiency of asRNA to trigger RNAi in vivo and in vitro. Finally, a nuclease (triangles) would cleave the mRNA.

Figure 12 is a bar graph showing sequence-specific gene silencing by 21-23 nt fragments. Ratio of luciferase activity after targeting of Pp-Luc and Rr-Luc mRNA by 5 nM Pp-Luc or Rr-Luc dsRNA (500 bp) or 21-23 nt fragments isolated from a previous incubation of the respective dsRNA in Drosophila lysate. The amount of isolated 21-23 mers present in the incubation reaction correspond to approximately the same amount of 21-23 mers generated during an incubation reaction with 5 nM 500 bp dsRNA. The data are average values of 3 trials and the standard deviation is given by error bars. Luciferase activity was normalized to the buffer control.

Figure 13A illustrates the purification of RNA fragments on a Superdex HR 200 10/30 gel filtration column (Pharmacia) using the method described in Example 4. dsRNA was 32P-labeled, and the radioactivity recovered in each column fraction is graphed. The fractions were also analyzed by denaturing gel electrophoresis (inset).

Figure 13B demonstrates the ability of the Rr-luciferase RNA, after incubation in the Drosophila lysate and fractionation as in Fig. 13A, to mediate sequence-specific

interference with the expression of a Rr-luciferase target mRNA. One microliter of each resuspended fraction was tested in a 10 microliter in vitro RNAi reaction (see Example 1). This procedure yields a concentration of RNA in the standard in vitro RNAi reaction that is approximately equal to the concentration of that RNA species in the original reaction prior to loading on the column. Relative luminescence per second has been normalized to the average value of the two buffer controls.

Figure 13C is the specificity control for Fig 13B. It demonstrates that the fractionated RNA of Fig 13B does not efficiently mediate sequence-specific interference with the expression of a Pp-luciferase mRNA. Assays are as in Fig 13B.

Figures 14A and 14B are schematic representations of reporter constructs and siRNA duplexes. Figure 14A illustrates the firefly (*Pp*-luc) and sea pansy (*Rr*-luc) luciferase reporter gene regions from plasmids pGL2-Control, pGL3-Control, and pRL-TK (Promega). SV40 regulatory elements, the HSV thymidine kinase promoter, and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase expression from pGL2 is approximately 10-fold lower than from pGL3 in transfected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes. Figure 14B shows the sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2 (SEQ ID Nos: 10 and 11), GL3 (SEQ ID Nos: 12 and 13), and RL (SEQ ID Nos: 14 and 15) luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only 3 single nucleotide substitutions (boxed in gray). As unspecific control, a duplex with the inverted GL2 sequence, invGL2 (SEQ ID Nos: 16 and 17), was synthesized. The 2 nt 3' overhang of 2'-deoxythymidine is indicated as TT; uGL2 (SEQ ID Nos: 18 and 19) is similar to GL2 siRNA but contains ribo-uridine 3' overhangs.

Figures 15A-15J are graphs showing RNA interference by siRNA duplexes. Ratios of target to control luciferase were normalized to a buffer control (bu, black bars); gray bars indicate ratios of *Photinus pyralis* (*Pp*-luc) GL2 or GL3 luciferase to *Renilla reniformis* (*Rr*-luc) RL luciferase (left axis), white bars indicate RL to GL2 or

GL3 ratios (right axis). Figures 15A, 15C, 15E, 15G, and 15I show results of experiments performed with the combination of pGL2-Control and pRL-TK reporter plasmids, Figures 15B, 15D, 15F, 15H, and 15J with pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of *Pp*-luc/*Rr*-luc for the buffer control (bu) varied between 0.5 and 10 for pGL2/pRL, and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and between the various cell lines tested. The plotted data were averaged from three independent experiments ± S.D.

Figures 16A-16F are graphs showing the effects of 21 nt siRNAs, 50 bp, and 500 bp dsRNAs on luciferase expression in HeLa cells. The exact length of the long dsRNAs is indicated below the bars. Figures 16A, 16C, and 16E describe experiments performed with pGL2-Control and pRL-TK reporter plasmids, Figures 16B, 16D, and 16F with pGL3-Control and pRL-TK reporter plasmids. The data were averaged from two independent experiments ± S.D. Figures 16A, 16B, Absolute *Pp*-luc expression, plotted in arbitrary luminescence units. Figure 16C, 16D, Rr-luc expression, plotted in arbitrary luminescence units. Figures 16E, 16F, Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (bu, black bars); the luminescence ratios for 50 or 500 bp dsRNAs were normalized to the respective ratios observed for 50 and 500 bp dsRNA from humanized GFP (hG, black bars). It should be noted, that the overall differences in sequence between the 49 and 484 bp dsRNAs targeting GL2 and GL3 are not sufficient to confer specificity between GL2 and GL3 targets (43 nt uninterrupted identity in 49 bp segment, 239 nt longest uninterrupted identity in 484 bp segment) (Parrish, S., *et al., Mol. Cell, 6:*1077-1087 (2000)).

DETAILED DESCRIPTION OF THE INVENTION

Double-stranded (dsRNA) directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). The process is known to occur in a wide variety of organisms, including embryos of mammals and other vertebrates.

Using the Drosophila in vitro system described herein, it has been demonstrated that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length, and furthermore, that when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of longer dsRNA. Thus, these 21-23 nt fragments are sequence-specific mediators of RNA degradation. A molecular signal, which may be the specific length of the fragments, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or recombinantly produced or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells. Use of long dsRNAs in mammalian cells to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. Specific targeting of a particular gene function, which is possible with 21-23 nt fragments of the present invention, is useful in functional genomic and therapeutic applications.

In particular, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that mediate RNAi. In one embodiment, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that direct cleavage of specific mRNA to which they correspond. The 21-23 nt RNA molecules of the present invention can also comprise a 3' hydroxyl group. The 21-23 nt RNA molecules can be single-stranded or double stranded (as two 21-23 nt RNAs); such molecules can be blunt ended or comprise overhanging ends (*e.g.*, 5', 3'). In specific embodiments, the RNA molecule is double stranded and either blunt ended or comprises overhanging ends (as two 21-23 nt RNAs).

In one embodiment, at least one strand of the RNA molecule has a 3' overhang from about 1 to about 6 nucleotides (*e.g.*, pyrimidine nucleotides, purine nucleotides) in length. In other embodiments, the 3' overhang is from about 1 to about 5 nucleotides, from about 1 to about 3 nucleotides and from about 2 to about 4 nucleotides in length. In one embodiment the RNA molecule is double stranded, one strand has a 3' overhang

and the other strand can be blunt-ended or have an overhang. In the embodiment in which the RNA molecule is double stranded and both strands comprise an overhang, the length of the overhangs may be the same or different for each strand. In a particular embodiment, the RNA of the present invention comprises 21 nucleotide strands which are paired and which have overhangs of from about 1 to about 3, particularly about 2, nucleotides on both 3' ends of the RNA. In order to further enhance the stability of the RNA of the present invention, the 3' overhangs can be stabilized against degradation. In one embodiment, the RNA is stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, *e.g.,* substitution of uridine 2 nucleotide 3' overhangs by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNAi. The absence of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium.

The 21-23 nt RNA molecules of the present invention can be obtained using a number of techniques known to those of skill in the art. For example, the RNA can be chemically synthesized or recombinantly produced using methods known in the art. The 21-23 nt RNAs can also be obtained using the Drosophila in vitro system described herein. Use of the Drosophila in vitro system entails combining dsRNA with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA of about 21 to about 23 nucleotides. The Drosophila in vitro system can also be used to obtain RNA of about 21 to about 23 nucleotides in length which mediates RNA interference of the mRNA of a particular gene (e.g., oncogene, viral gene). In this embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to the RNA of about 21 to about 23 nucleotides. As shown herein, 21-23 nt RNA mediates RNAi of the mRNA to be degraded. The present

invention also relates to the 21-23 nt RNA molecules produced by the methods described herein.

In one embodiment, the methods described herein are used to identify or obtain 21-23 nt RNA molecules that are useful as sequence-specific mediators of RNA degradation and, thus, for inhibiting mRNAs, such as human mRNAs, that encode products associated with or causative of a disease or an undesirable condition. For example, production of an oncoprotein or viral protein can be inhibited in humans in order to prevent the disease or condition from occurring, limit the extent to which it occurs or reverse it. If the sequence of the gene to be targeted in humans is known, 21-23 nt RNAs can be produced and tested for their ability to mediate RNAi in a cell, such as a human or other primate cell. Those 21-23 nt human RNA molecules shown to mediate RNAi can be tested, if desired, in an appropriate animal model to further assess their in vivo effectiveness. Additional copies of 21-23 nt RNAs shown to mediate RNAi can be produced by the methods described herein.

The method of obtaining the 21-23 nt RNA sequence using the Drosophila in vitro system can further comprise isolating the RNA sequence from the combination. The 21-23 nt RNA molecules can be isolated using a number of techniques known to those of skill in the art. For example, gel electrophoresis can be used to separate 21-23 nt RNAs from the combination, gel slices comprising the RNA sequences removed and RNAs eluted from the gel slices. Alternatively, non-denaturing methods, such as non-denaturing column chromatography, can be used to isolate the RNA produced. In addition, chromatography (e.g., size exclusion chromatography), glycerol gradient centrifugation, affinity purification with antibody can be used to isolate 21-23 nt RNAs. The RNA-protein complex isolated from the Drosophila in vitro system can also be used directly in the methods described herein (e.g., method of mediating RNAi of mRNA of a gene). Soluble extracts derived from Drosophila embryo that mediate or RNAi are encompassed by the invention. The soluble Drosophila extract can be obtained in a variety of ways. For example, the soluble extract can be obtained from syncytial blastoderm Drosophila embryos as described in Examples 1, 2, and 3. Soluble

extracts can be derived from other cells in which RNAi occurs. Alternatively, soluble extracts can be obtained from a cell that does not carry out RNAi. In this instance, the factors needed to mediate RNAi can be introduced into such a cell and the soluble extract is then obtained. The components of the extract can also be chemically synthesized and/or combined using methods known in the art.

Any dsRNA can be used in the methods of the present invention, provided that it has sufficient homology to the targeted gene to mediate RNAi. The sequence of the dsRNA for use in the methods of the present invention need not be known. Alternatively, the dsRNA for use in the present invention can correspond to a known sequence, such as that of an entire gene (one or more) or portion thereof. There is no upper limit on the length of the dsRNA that can be used. For example, the dsRNA can range from about 21 base pairs (bp) of the gene to the full length of the gene or more. In one embodiment, the dsRNA used in the methods of the present invention is about 1000 bp in length. In another embodiment, the dsRNA is about 500 bp in length. In yet another embodiment, the dsRNA is about 22 bp in length.

The 21 to 23 nt RNAs described herein can be used in a variety of ways. For example, the 21 to 23 nt RNA molecules can be used to mediate RNA interference of mRNA of a gene in a cell or organism. In a specific embodiment, the 21 to 23 nt RNA is introduced into human cells or a human in order to mediate RNA interference in the cells or in cells in the individual, such as to prevent or treat a disease or undesirable condition. In this method, a gene (or genes) that cause or contribute to the disease or undesirable condition is targeted and the corresponding mRNA (the transcriptional product of the targeted gene) is degraded by RNAi. In this embodiment, an RNA of about 21 to about 23 nucleotides that targets the corresponding mRNA (the mRNA of the targeted gene) for degradation is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of the corresponding mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism. In a particular embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a

sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides. The 21 to 23 nt RNA is then isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the gene in the cell or organism. In the event that the 21-23nt RNA is introduced into a cell in which RNAi, does not normally occur, the factors needed to mediate RNAi are introduced into such a cell or the expression of the needed factors is induced in such a cell. Alternatively, 21 to 23 nt RNA produced by other methods (e.g., chemical synthesis, recombinant DNA production) to have a composition the same as or sufficiently similar to a 21 to 23 nt RNA known to mediate RNAi can be similarly used to mediate RNAi. Such 21 to 23 nt RNAs can be altered by addition, deletion, substitution or modification of one or more nucleotides and/or can comprise non-nucleotide materials. A further embodiment of this invention is an ex vivo method of treating cells from an individual to degrade a gene(s) that causes or is associated with a disease or undesirable condition, such as leukemia or AIDS. In this embodiment, cells to be treated are obtained from the individual using known methods (e.g., phlebotomy or collection of bone marrow) and 21-23 nt RNAs that mediate degradation of the corresponding mRNA(s) are introduced into the cells, which are then re-introduced into the individual. If necessary, biochemical components needed for RNAi to occur can also be introduced into the cells.

The mRNA of any gene can be targeted for degradation using the methods of mediating interference of mRNA described herein. For example, any cellular or viral mRNA, can be targeted, and, as a result, the encoded protein (e.g., an oncoprotein, a viral protein), expression will be diminished. In addition, the mRNA of any protein associated with/causative of a disease or undesirable condition can be targeted for degradation using the methods described herein.

The present invention also relates to a method of examining the function of a gene in a cell or organism. In one embodiment, an RNA sequence of about 21 to about

23 nucleotides that targets mRNA of the gene for degradation is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of mRNA of the gene occurs. The phenotype of the cell or organism is then observed and compared to an appropriate control, thereby providing information about the function of the gene. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract derived from Drosophila embryo under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of the mRNA of the gene occurs. The phenotype of the cell or organism is then observed and compared to an appropriate control, thereby identifying the function of the gene.

A further aspect of this invention is a method of assessing the ability of 21-23 nt RNAs to mediate RNAi and, particularly, determining which 21-23 nt RNA(s) most efficiently mediate RNAi. In one embodiment of the method, dsRNA corresponding to a sequence of an mRNA to be degraded is combined with detectably labeled (e.g., end-labeled, such as radiolabeled) mRNA and the soluble extract of this invention, thereby producing a combination. The combination is maintained under conditions under which the double-stranded RNA is processed and the mRNA is degraded. The sites of the most effective cleavage are mapped by comparing the migration of the labeled mRNA cleavage products to markers of known length. 21 mers spanning these sites are then designed and tested for their efficiency in mediating RNAi.

Alternatively, the extract of the present invention can be used to determine whether there is a particular segment or particular segments of the mRNA corresponding to a gene which are more efficiently targeted by RNAi than other regions and, thus, can be especially useful target sites. In one embodiment, dsRNA corresponding to a sequence of a gene to be degraded, labeled mRNA of the gene is combined with a soluble extract that mediates RNAi, thereby producing a combination. The resulting combination is maintained under conditions under which the dsRNA is

degraded and the sites on the mRNA that are most efficiently cleaved are identified, using known methods, such as comparison to known size standards on a sequencing gel.

OVERVIEW OF EXAMPLES

Biochemical analysis of RNAi has become possible with the development of the in vitro Drosophila embryo lysate that recapitulates dsRNA-dependent silencing of gene expression described in Example 1 (Tuschl et al., Genes Dev., 13:3191-7 (1999)). In the in vitro system, dsRNA, but not sense or asRNA, targets a corresponding mRNA for degradation, yet does not affect the stability of an unrelated control mRNA. Furthermore, pre-incubation of the dsRNA in the lysate potentiates its activity for target mRNA degradation, suggesting that the dsRNA must be converted to an active form by binding proteins in the extract or by covalent modification (Tuschl et al., Genes Dev., 13:3191-7 (1999)).

The development of a cell-free system from syncytial blastoderm Drosophila embryos that recapitulates many of the features of RNAi is described herein. The interference observed in this reaction is sequence-specific, is promoted by dsRNA, but not by single-stranded RNA, functions by specific mRNA degradation, requires a minimum length of dsRNA and is most efficient with long dsRNA. Furthermore, preincubation of dsRNA potentiates its activity. These results demonstrate that RNAi is mediated by sequence specific processes in soluble reactions.

As described in Example 2, the in vitro system was used to analyze the requirements of RNAi and to determine the fate of the dsRNA and the mRNA. RNAi in vitro requires ATP, but does not require either mRNA translation or recognition of the 7-methyl-guanosine cap of the targeted mRNA. The dsRNA, but not single-stranded RNA, is processed in vitro to a population of 21-23 nt species. Deamination of adenosines within the dsRNA does not appear to be required for formation of the 21-23 nt RNAs. As described herein, the mRNA is cleaved only in the region corresponding to the sequence of the dsRNA and that the mRNA is cleaved at 21-23 nt intervals, strongly indicating that the 21-23 nt fragments from the dsRNA are targeting the

cleavage of the mRNA. Furthermore, as described in Examples 3 and 4, when the 21-23 nt fragments are purified and added back to the soluble extract, they mediate RNA.

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

Example 1 Targeted mRNA degradation by double-stranded RNA in vitro

Materials and Methods

RNAs

Rr-Luc mRNA consisted of the 926 nt Rr luciferase coding sequence flanked by 25 nt of 5' untranslated sequence from the pSP64 plasmid polylinker and 25 nt of 3' untranslated sequence consisting of 19 nt of pSP64 plasmid polylinker sequence followed by a 6 nt Sac I site. Pp-Luc mRNA contained the 1653 nt Pp luciferase coding sequence with a Kpn I site introduced immediately before the Pp luciferase stop codon. The Pp coding sequence was flanked by 5' untranslated sequences consisting of 21 nt of pSP64 plasmid polylinker followed by the 512 nt of the 5' untranslated region (UTR) from the Drosophila hunchback mRNA and 3' untranslated sequences consisting of the 562 nt hunchback 3' UTR followed by a 6 nt Sac I site. The hunchback 3' UTR sequences used contained six G-to-U mutations that disrupt function of the Nanos Response Elements in vivo and in vitro. Both reporter mRNAs terminated in a 25 nt poly(A) tail encoded in the transcribed plasmid. For both Rr-Luc and Pp -Luc mRNAs, the transcripts were generated by run-off transcription from plasmid templates cleaved at an Nsi I site that immediately followed the 25 nt encoded poly(A) tail. To ensure that the transcripts ended with a poly(A) tail, the Nsi I-cleaved transcription templates were resected with T4 DNA Polymerase in the presence of dNTPs. The SP6 mMessage mMachine kit (Ambion) was used for in vitro transcription. Using this kit, about 80% of the resulting transcripts are 7-methyl guanosine capped. ^{32}P-radiolabeling was accomplished by including α-^{32}P-UTP in the transcription reaction.

For Pp -Luc, ss, as, and dsRNA corresponded to positions 93 to 597 relative to the start of translation, yielding a 505 bp dsRNA. For Rr -Luc, ss, as, and dsRNA corresponded to positions 118 to 618 relative to the start of translation, yielding a 501 bp dsRNA. The Drosophila nanos competitor dsRNA corresponded to positions 122 to 629 relative to the start of translation, yielding a 508 bp dsRNA. ssRNA, asRNA, and dsRNA (diagrammed in Figure 1) were transcribed in vitro with T7 RNA polymerase from templates generated by the polymerase chain reaction. After gel purification of the T7 RNA transcripts, residual DNA template was removed by treatment with RQ1 DNase (Promega). The RNA was then extracted with phenol and chloroform, and then precipitated and dissolved in water.

RNA annealing and native gel electrophoresis.

ssRNA and asRNA (0.5 μM) in 10 mM Tris-HCl (pH 7.5) with 20 mM NaCl were heated to 95 ° C for 1 min then cooled and annealed at room temperature for 12 to 16 h. The RNAs were precipitated and resuspended in lysis buffer (below). To monitor annealing, RNAs were electrophoresed in a 2% agarose gel in TBE buffer and stained with ethidium bromide (Sambrook et al., Molecular Cloning. Cold Spring Harbor Laboratory Press, Plainview, NY. (1989)).

Lysate preparation

Zero- to two-hour old embryos from Oregon R flies were collected on yeasted molasses agar at 25°C. Embryos were dechorionated for 4 to 5 min in 50% (v/v) bleach, washed with water, blotted dry, and transferred to a chilled Potter-Elvehjem tissue grinder (Kontes). Embryos were lysed at 4°C in one ml of lysis buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) containing 5 mM dithiothreitol (DTT) and 1 mg/ml Pefabloc SC (Boehringer-Mannheim) per gram of damp embryos. The lysate was centrifuged for 25 min at 14,500 x g at 4°C, and the supernatant flash frozen in aliquots in liquid nitrogen and stored at -80°C.

Reaction conditions

Lysate preparation and reaction conditions were derived from those described by Hussain and Leibowitz (Hussain and Leibowitz, Gene 46:13-23 (1986)). Reactions contained 50% (v/v) lysate, mRNAs (10 to 50 pM final concentration), and 10% (v/v) lysis buffer containing the ssRNA, asRNA, or dsRNA (10 nM final concentration). Each reaction also contained 10 mM creatine phosphate, 10 μg/ml creatine phosphokinase, 100 μM GTP, 100 μM UTP, 100 μM CTP, 500 μM ATP, 5 μM DTT, 0.1 U/mL RNasin (Promega), and 100 μM of each amino acid. The final concentration of potassium acetate was adjusted to 100 mM. For standard conditions, the reactions were assembled on ice and then pre-incubated at 25° C for 10 min before adding mRNA. After adding mRNAs, the incubation was continued for an additional 60 min. The 10 min preincubation step was omitted for the experiments in Figures 3A-3C and 5A-5C. Reactions were quenched with four volumes of 1.25x Passive Lysis Buffer (Promega). Pp and Rr luciferase activity was detected in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory) using the Dual-Luciferase Reporter Assay System (Promega).

RNA stability

Reactions with ^{32}P-radiolabeled mRNA were quenched by the addition of 40 volumes of 2x PK buffer (200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% w/v sodium dodecyl sulfate). Proteinase K (E.M. Merck; dissolved in water) was added to a final concentration of 465 μg/ml. The reactions were then incubated for 15 min at 65° C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with an equal volume of isopropanol. Reactions were analyzed by electrophoresis in a formaldehyde/agarose (0.8% w/v) gel (Sambrook et al., Molecular Cloning. Cold Spring Harbor Laboratory Press, Plainview, NY. (1989)). Radioactivity was detected by exposing the agarose gel [dried under vacuum onto Nytran Plus membrane (Amersham)] to an image plate (Fujix) and quantified using a Fujix Bas 2000 and Image Gauge 3.0 (Fujix) software.

Commercial lysates

Untreated rabbit reticulocyte lysate (Ambion) and wheat germ extract (Ambion) reactions were assembled according to the manufacturer's directions. dsRNA was incubated in the lysate at 27°C (wheat germ) or 30°C (reticulocyte lysate) for 10 min prior to the addition of mRNAs.

Results and Discussion

To evaluate if dsRNA could specifically block gene expression in vitro, reporter mRNAs derived from two different luciferase genes that are unrelated both in sequence and in luciferin substrate specificity were used: Renilla reniformis (sea pansy) luciferase (Rr-Luc) and Photuris pennsylvanica (firefly) luciferase (Pp-Luc). dsRNA generated from one gene was used to target that luciferase mRNA whereas the other luciferase mRNA was an internal control co-translated in the same reaction. dsRNAs of approximately 500 bp were prepared by transcription of polymerase-chain reaction products from the Rr-Luc and Pp-Luc genes. Each dsRNA began ~100 bp downstream of the start of translation (Figure 1). Sense (ss) and anti-sense (as) RNA were transcribed in vitro and annealed to each other to produce the dsRNA. Native gel electrophoresis of the individual Rr 501 and Pp 505 nt as RNA and ssRNA used to form the Rr and Pp dsRNAs was preformed. The ssRNA, asRNA, and dsRNAs were each tested for their ability to block specifically expression of their cognate mRNA but not the expression of the unrelated internal control mRNA.

The ssRNA, asRNA, or dsRNA was incubated for 10 min in a reaction containing Drosophila embryo lysate, then both Pp-Luc and Rr-Luc mRNAs were added and the incubation continued for an additional 60 min. The Drosophila embryo lysate efficiently translates exogenously transcribed mRNA under the conditions used. The amounts of Pp-Luc and Rr-Luc enzyme activities were measured and were used to calculate ratios of either Pp-Luc/Rr-Luc (Figure 2A) or Rr-Luc/Pp-Luc (Figure 2B). To facilitate comparison of different experiments, the ratios from each experiment were

normalized to the ratio observed for a control in which buffer was added to the reaction in place of ssRNA, asRNA, or dsRNA.

Figure 2A shows that a 10 nM concentration of the 505 bp dsRNA identical to a portion of the sequence of the Pp-Luc gene specifically inhibited expression of the Pp-Luc mRNA but did not affect expression of the Rr-Luc internal control. Neither ssRNA nor asRNA affected expression of Pp-Luc or the Rr-Luc internal control. Thus, Pp-Luc expression was specifically inhibited by its cognate dsRNA. Conversely, a 10 nM concentration of the 501 bp dsRNA directed against the Rr-Luc mRNA specifically inhibited Rr-Luc expression but not that of the Pp-Luc internal control (Figure 2B). Again, comparable levels of ssRNA or asRNA had little or no effect on expression of either reporter mRNA. On average, dsRNA reduced specific luciferase expression by 70% in these experiments, in which luciferase activity was measured after 1 h incubation. In other experiments in which the translational capacity of the reaction was replenished by the addition of fresh lysate and reaction components, a further reduction in targeted luciferase activity relative to the internal control was observed.

The ability of dsRNA but not asRNA to inhibit gene expression in these lysates is not merely a consequence of the greater stability of the dsRNA (half-life about 2 h) relative to the single-stranded RNAs (half-life ~ 10 min). ssRNA and asRNA transcribed with a 7-methyl guanosine cap were as stable in the lysate as uncapped dsRNA, but do not inhibit gene expression. In contrast, dsRNA formed from the capped ssRNA and asRNA specifically blocks expression of the targeted mRNA.

Effective RNAi in Drosophila requires the injection of about 0.2 fmol of dsRNA into a syncytial blastoderm embryo (Kennerdell and Carthew, Cell 95:1017-1026 (1998); Carthew, www1.pitt.edu/~carthew/manual/RNAi_Protocol.html (1999)). Since the average volume of a Drosophila embryo is approximately 7.3 nl, this corresponds to an intracellular concentration of about 25 nM (Mazur et al., Cryobiology 25:543-544 (1988)). Gene expression in the Drosophila lysate was inhibited by a comparable concentration of dsRNA (10 nM), but lowering the dsRNA concentration ten-fold decreased the amount of specific interference. Ten nanomolar dsRNA corresponds to a

200-fold excess of dsRNA over target mRNA added to the lysate. To test if this excess of dsRNA might reflect a time- and/or concentration-dependent step in which the input dsRNA was converted to a form active for gene-specific interference, the effect of preincubation of the dsRNA on its ability to inhibit expression of its cognate mRNA was examined. Because the translational capacity of the lysates is significantly reduced after 30 min of incubation at 25°C (unpublished observations), it was desired to ensure that all factors necessary for RNAi remained active throughout the pre-incubation period. Therefore, every 30 min, a reaction containing dsRNA and lysate was mixed with a fresh reaction containing unincubated lysate (Figure 3A). After six successive serial transfers spanning 3 hours of preincubation, the dsRNA, now diluted 64-fold relative to its original concentration, was incubated with lysate and 50 pM of target mRNA for 60 min. Finally, the Pp-Luc and Rr-Luc enzyme levels were measured. For comparison, the input amount of dsRNA (10 nM) was diluted 32-fold in buffer, and its capacity to generate gene-specific dsRNA interference in the absence of any preincubation step was assessed.

The preincubation of the dsRNA in lysate significantly potentiated its capacity to inhibit specific gene expression. Whereas the dsRNA diluted 32-fold showed no effect, the preincubated dsRNA was, within experimental error, as potent as undiluted dsRNA, despite having undergone a 64-fold dilution. Potentiation of the dsRNA by preincubation was observed for dsRNAs targeting both the Pp-Luc mRNA (Figure 3B) and the Rr-Luc mRNA (Figure 3C). Taking into account the 64-fold dilution, the activation conferred by preincubation allowed a 156 pM concentration of dsRNA to inhibit 50 pM target mRNA. Further, dilution of the "activated" dsRNA may be effective but has not been tested. We note that although both dsRNAs tested were activated by the preincubation procedure, each fully retained its specificity to interfere with expression only of the mRNA to which it is homologous. Further study of the reactions may provide a route to identifying the mechanism of dsRNA potentiation.

One possible explanation for the observation that preincubation of the dsRNA enhances its capacity to inhibit gene expression in these lysates is that specific factors

either modify and/or associate with the dsRNA. Accordingly, the addition of increasing amounts of dsRNA to the reaction might titrate such factors and decrease the amount of gene-specific interference caused by a second dsRNA of unrelated sequence. For both Pp-Luc mRNA and Rr-Luc mRNA, addition of increasing concentrations of the unrelated Drosophila nanos dsRNA to the reaction decreased the amount of gene-specific interference caused by dsRNA targeting the reporter mRNA (Figure 4). None of the tested concentrations of nanos dsRNA affected the levels of translation of the untargeted mRNA, demonstrating that the nanos dsRNA specifically titrated factors involved in gene-specific interference and not components of the translational machinery. The limiting factor(s) was titrated by addition of approximately 1000 nM dsRNA, a 200-fold excess over the 5 nM of dsRNA used to produce specific interference.

Interference in vitro might reflect either a specific inhibition of mRNA translation or the targeted destruction of the specific mRNA. To distinguish these two possibilities, the fates of the Pp-Luc and Rr-Luc mRNAs were examined directly using ^{32}P-radiolabeled substrates. Stability of 10 nM Pp-Luc mRNA or Rr-Luc mRNA incubated in lysate with either buffer or 505 bp Pp-dsRNA (10 nM). Samples were deproteinized after the indicated times and the ^{32}P-radiolabeled mRNAs were then resolved by denaturing gel electrophoresis. In the absence of dsRNA, both the Pp-Luc and Rr-Luc mRNAs were stable in the lysates, with ~ 75% of the input mRNA remaining after 3 h of incubation. (About 25% of the input mRNA is rapidly degraded in the reaction and likely represents uncapped mRNA generated by the in vitro transcription process.) In the presence of dsRNA (10 nM, 505 bp) targeting the Pp-Luc mRNA, less than 15% of the Pp-Luc mRNA remained after 3 h (Figure 5A). As expected, the Rr-Luc mRNA remained stable in the presence of the dsRNA targeting Pp-Luc mRNA. Conversely, dsRNA (10 nM, 501 bp) targeting the Rr-Luc mRNA caused the destruction of the Rr-Luc mRNA but had no effect on the stability of Pp-Luc mRNA (Figure 5B). Thus, the dsRNA specifically caused accelerated decay of the mRNA to which it is homologous with no effect on the stability of the unrelated control

mRNA. This finding indicates that in vivo, at least in Drosophila, the effect of dsRNA is to directly destabilize the target mRNA, not to change the subcellular localization of the mRNA, for example, by causing it to be specifically retained in the nucleus, resulting in non-specific degradation.

These results are consistent with the observation that RNAi leads to reduced cytoplasmic mRNA levels in vivo, as measured by in situ hybridization (Montgomery et al., Proc. Natl. Acad. Sci. USA 95:15502-15507 (1998)) and Northern blotting (Ngo et al., Proc. Natl. Acad. Sci. USA 95:14687-14692 (1998)). Northern blot analyses in trypanosomes and hydra suggest that dsRNA typically decreases mRNA levels by less than 90% (Ngo et al., Proc. Natl. Acad. Sci. USA 95:14687-14692 (1998); Lohmann et al., Dev. Biol. 214:211-214 (1999)). The data presented here show that in vitro mRNA levels are reduced 65 to 85% after three hours incubation, an effect comparable with observations in vivo. They also agree with the finding that RNAi in C. elegans is post-transcriptional (Montgomery et al., Proc. Natl. Acad. Sci. USA 95:15502-15507 (1998)). The simplest explanation for the specific effects on protein synthesis is that it reflects the accelerated rate of RNA decay. However, the results do not exclude independent but specific effects on translation as well as stability.

In vivo, RNAi appears to require a minimum length of dsRNA (Ngo et al., Proc. Natl. Acad. Sci., USA, 95:14687-14692 (1998)). The ability of RNA duplexes of lengths 49 bp, 149 bp, 505 bp, and 997 bp (diagrammed in Figure 1) to target the degradation of the Pp-Luc mRNA in vitro was assessed. In good agreement with in vivo observations, the 49 bp dsRNA was ineffective in vitro, while the 149 bp dsRNA enhanced mRNA decay only slightly, and both the 505 and 997 bp dsRNAs caused robust mRNA degradation (Figure 5C). 50bp dsRNA targeting other portions of the mRNA cause detectable mRNA degradation, though not as robust as that seen for 500bp dsRNA. Thus, although some short dsRNA do not mediate RNAi, others of approximately the same length, but different composition, will be able to do so.

Whether the gene-specific interference observed in Drosophila lysates was a general property of cell-free translation systems was examined. The effects of dsRNAs

on expression of Pp-Luc and Rr-Luc mRNA were examined in commercially available wheat germ extracts and rabbit reticulocyte lysates. There was no effect of addition of 10 nM of either ssRNA, asRNA, or dsRNA on the expression of either mRNA reporter in wheat germ extracts. In contrast, the addition of 10 nM of dsRNA to the rabbit reticulocyte lysate caused a profound and rapid, non-specific decrease in mRNA stability. For example, addition of Rr-Luc dsRNA caused degradation of both Rr-Luc and Pp-Luc mRNAs within 15 min. The same non-specific effect was observed upon addition of Pp-Luc dsRNA. The non-specific destruction of mRNA induced by the addition of dsRNA to the rabbit reticulocyte lysate presumably reflects the previously observed activation of RNase L by dsRNA (Clemens and Williams, Cell 13:565-572 (1978); Williams et al., Nucleic Acids Res. 6:1335-1350 (1979); Zhou et al., Cell 72:753-765 (1993); Matthews, Interactions between Viruses and the Cellular Machinery for Protein Synthesis. In Translational Control (eds. J. Hershey, M. Mathews and N. Sonenberg), pp. 505-548. Cold Spring Harbor Laboratory Press, Plainview, NY. (1996)). Mouse cell lines lacking dsRNA-induced anti-viral pathways have recently been described (Zhou et al., Virology 258:435-440 (1999)) and may be useful in the search for mammalian RNAi. Although RNAi is known to exist in some mammalian cells (Wianny and Zernicka-Goetz Nat. Cell Biol. 2: 70-75 (2000)), in many mammalian cell types its presence is likely obscured by the rapid induction by dsRNA of non-specific anti-viral responses.

dsRNA-targeted destruction of specific mRNA is characteristic of RNAi, which has been observed in vivo in many organisms, including Drosophila. The system described above recapitulates in a reaction in vitro many aspects of RNAi. The targeted mRNA is specifically degraded whereas unrelated control mRNAs present in the same solution are not affected. The process is most efficient with dsRNAs greater than 150 bp in length. The dsRNA-specific degradation reaction in vitro is probably general to many, if not all, mRNAs since it was observed using two unrelated genes.

The magnitude of the effects on mRNA stability in vitro described herein are comparable with those reported in vivo (Ngo et al., Proc. Natl. Acad. Sci., USA,

95:14687-14692 (1998); Lohmann et al., Dev. Biol., 214:211-214 (1999). However, the reaction in vitro requires an excess of dsRNA relative to mRNA. In contrast, a few molecules of dsRNA per cell can inhibit gene expression in vivo (Fire et al., Nature, 391: 806-811 (1998); Kennerdell and Carthew, Cell, 95:1017-1026 (1998)). The difference between the stoichiometry of dsRNA to target mRNA in vivo and in vitro should not be surprising in that most in vitro reactions are less efficient than their corresponding in vivo processes. Interestringly, incubation of the dsRNA in the lysate greatly potentiated its activity for RNAi, indicating that it is either modified or becomes associated with other factors or both. Perhaps a small number of molecules is effective in inhibiting the targeted mRNA in vivo because the injected dsRNA has been activated by a process similar to that reported here for RNAi in Drosophila lysates.

Example 2 Double-Stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals

Methods and Material

In vitro RNAi

In vitro RNAi reactions and lysate preparation were as described in Example 1 (Tuschl et al., Genes Dev., 13:3191-7 (1999)) except that the reaction contained 0.03 g/ml creatine kinase, 25 μM creatine phosphate (Fluka), and 1 mM ATP. Creatine phosphate was freshly dissolved at 500 mM in water for each experiment. GTP was omitted from the reactions, except in Figures 2 and 3.

RNA Synthesis.

Pp-luc and Rr-luc mRNAs and Pp- and Rr-dsRNAs (including dsRNA 'B' in Figure 6) were synthesized by in vitro transcription as described previously (Tuschl et al., Genes Dev., 13:3191-7 (1999)). To generate transcription templates for dsRNA 'C,' the 5′ sense RNA primer was gcgtaatacgactcactataGAACAAAGGAAACGGATGAT (SEQ ID NO: 2) and the 3′ sense RNA primer was GAAGAAGTTATTCTCCAAAA (SEQ ID NO: 3); the 5′ asRNA primer was

gcgtaatacgactcactataGAAGAAGTTATTCTCCAAAA (SEQ ID NO: 4)and the 3′ asRNA primer was GAACAAAGGAAACGGATGAT (SEQ ID NO: 5). For dsRNA 'A' the 5′ sense RNA primer was gcgtaatacgactcactataGTAGCGCGGTGTATTATACC (SEQ ID NO: 6)and the 3′ sense RNA primer was GTACAACGTCAGGTTTACCA (SEQ ID NO: 7); the 5′ asRNA primer was gcgtaatacgactcactataGTACAACGTCAGGTTTACCA (SEQ ID NO: 8)and the 3′ asRNA primer was GTAGCGCGGTGTATTATACC (SEQ ID NO: 9) (lowercase, T7 promoter sequence).

mRNAs were 5′-end-labeled using guanylyl transferase (Gibco/BRL), S-adenosyl methionine (Sigma), and α-^{32}P-GTP (3000 Ci/mmol; New England Nuclear) according to the manufacturer's directions. Radiolabeled RNAs were purified by poly(A) selection using the Poly(A) Tract III kit (Promega). Nonradioactive 7-methyl-guanosine- and adenosine-capped RNAs were synthesized in in vitro transcription reactions with a 5-fold excess of 7-methyl-G(5′)ppp(5′)G or A(5′)ppp(5′)G relative to GTP. Cap analogs were purchased from New England Biolabs.

ATP depletion and Protein Synthesis Inhibition

ATP was depleted by incubating the lysate for 10 minutes at 25°C with 2 mM glucose and 0.1 U/ml hexokinase (Sigma). Protein synthesis inhibitors were purchased from Sigma and dissolved in absolute ethanol as 250-fold concentrated stocks. The final concentrations of inhibitors in the reaction were: anisomycin, 53 mg/ml; cycloheximide, 100 mg/ml; chloramphenicol, 100 mg/ml. Relative protein synthesis was determined by measuring the activity of Rr luciferase protein produced by translation of the Rr-luc mRNA in the RNAi reaction after 1 hour as described previously (Tuschl et al., Genes Dev., 13:3191-7 (1999)).

Analysis of dsRNA Processing

Internally α-^{32}P-ATP-labeled dsRNAs (505 bp Pp-luc or 501 Rr-luc) or 7-methyl-guanosine-capped Rr-luc antisense RNA (501 nt) were incubated at 5 nM final

concentration in the presence or absence of unlabeled mRNAs in Drosophila lysate for 2 hours in standard conditions. Reactions were stopped by the addition of 2x proteinase K buffer and deproteinized as described previously (Tuschl et al., Genes Dev., 13:3191-3197 (1999)). Products were analyzed by electrophoresis in 15% or 18% polyacrylamide sequencing gels. Length standards were generated by complete RNase T1 digestion of α-^{32}P-ATP-labeled 501 nt Rr-luc sense RNA and asRNA.

For analysis of mRNA cleavage, 5′-^{32}P-radiolabeled mRNA (described above) was incubated with dsRNA as described previously (Tuschl et al., Genes Dev., 13:3191-3197 (1999)) and analyzed by electrophoresis in 5% (Figure 5B) and 6% (Figure 6C) polyacrylamide sequencing gels. Length standards included commercially available RNA size standards (FMC Bioproducts) radiolabeled with guanylyl transferase as described above and partial base hydrolysis and RNase T1 ladders generated from the 5′-radiolabeled mRNA.

Deamination Assay

Internally α-^{32}P-ATP-labeled dsRNAs (5 nM) were incubated in Drosophila lysate for 2 hours at standard conditions. After deproteinization, samples were run on 12% sequencing gels to separate full-length dsRNAs from the 21-23 nt products. RNAs were eluted from the gel slices in 0.3 M NaCl overnight, ethanol-precipitated, collected by centrifugation, and redissolved in 20 µl water. The RNA was hydrolyzed into nucleoside 5 -phosphates with nuclease P1 (10 µl reaction containing 8 µl RNA in water, 30 mM KOAc pH 5.3, 10 mM $ZnSO_4$, 10 µg or 3 units nuclease P1, 3 hours, 50° C). Samples (1 ml) were co-spotted with non-radioactive 5 -mononucleotides [0.05 O.D. units (A_{260}) of pA, pC, pG, pI, and pU] on cellulose HPTLC plates (EM Merck) and separated in the first dimension in isobutyric acid/25% ammonia/water (66/1/33, v/v/v) and in the second dimension in 0.1M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol (100/60/2, v/w/v; Silberklang et al., 1979). Migration of the non-radioactive internal standards was determined by UV-shadowing.

Results and Discussion

RNAi Requires ATP

As described in Example 1, Drosophila embryo lysates faithfully recapitulate RNAi (Tuschl et al., Genes Dev., 13:3191-7 (1999)). Previously, dsRNA-mediated gene silencing was monitored by measuring the synthesis of luciferase protein from the targeted mRNA. Thus, these RNAi reactions contained an ATP-regenerating system, needed for the efficient translation of the mRNA. To test if ATP was, in fact, required for RNAi, the lysates were depleted for ATP by treatment with hexokinase and glucose, which converts ATP to ADP, and RNAi was monitored directly by following the fate of ^{32}P-radiolabeled Renilla reniformis luciferase (Rr-luc) mRNA (Figure 6). Treatment with hexokinase and glucose reduced the endogenous ATP level in the lysate from 250 μM to below 10 μM. ATP regeneration required both exogenous creatine phosphate and creatine kinase, which acts to transfer a high-energy phosphate from creatine phosphate to ADP. When ATP-depleted extracts were supplemented with either creatine phosphate or creatine kinase separately, no RNAi was observed. Therefore, RNAi requires ATP in vitro. When ATP, creatine phosphate, and creatine kinase were all added together to reactions containing the ATP-depleted lysate, dsRNA-dependent degradation of the Rr-luc mRNA was restored (Figure 6). The addition of exogenous ATP was not required for efficient RNAi in the depleted lysate, provided that both creatine phosphate and creatine kinase were present, demonstrating that the endogenous concentration (250 mM) of adenosine nucleotide is sufficient to support RNAi. RNAi with a Photinus pyralis luciferase (Pp-luc) mRNA was also ATP-dependent.

The stability of the Rr-luc mRNA in the absence of Rr-dsRNA was reduced in ATP-depleted lysates relative to that observed when the energy regenerating system was included, but decay of the mRNA under these conditions did not display the rapid decay kinetics characteristic of RNAi in vitro, nor did it generate the stable mRNA cleavage products characteristic of dsRNA-directed RNAi. These experiments do not establish if the ATP requirement for RNAi is direct, implicating ATP in one or more steps in the

RNAi mechanism, or indirect, reflecting a role for ATP in maintaining high concentrations of another nucleoside triphosphate in the lysate.

Translation Is Not Required for RNAi In Vitro

The requirement for ATP suggested that RNAi might be coupled to mRNA translation, a highly energy-dependent process. To test this possibility, various inhibitors of protein synthesis were added to the reaction by preparing a denaturing agarose-gel analysis of 5′-32P-radiolabeled Pp-luc mRNA after incubation for indicated times in a standard RNAi reaction with and without protein synthesis inhibitors. The eukaryotic translation inhibitors anisomycin, an inhibitor of initial peptide bond formation, cycloheximide, an inhibitor of peptide chain elongation, and puromycin, a tRNA mimic which causes premature termination of translation (Cundliffe, Antibiotic Inhibitors of Ribosome Function. In The Molecular Basis of Antibiotic Action, E. Gale, E. Cundliffe, P. Reynolds, M. Richmond and M. Warning, eds. (New York: Wiley), pp. 402-547. (1981)) were tested. Each of these inhibitors reduced protein synthesis in the Drosophila lysate by more than 1,900-fold (Figure 7A). In contrast, chloramphenicol, an inhibitor of Drosophila mitochondrial protein synthesis (Page and Orr-Weaver, Dev. Biol., 183:195-207 (1997)), had no effect on translation in the lysates (Figure 7A). Despite the presence of anisomycin, cycloheximide, or chloramphenicol, RNAi proceeded at normal efficiency. Puromycin also did not perturb efficient RNAi. Thus, protein synthesis is not required for RNAi in vitro.

Translational initiation is an ATP-dependent process that involves recognition of the 7-methyl guanosine cap of the mRNA (Kozak, Gene, 234:187-208 (1999); Merrick and Hershey, The Pathway and Mechanism of Eukaryotic Protein Synthesis. In Translational Control, J. Hershey, M. Mathews and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 31-69 (1996)). The Drosophila lysate used to support RNAi in vitro also recapitulates the cap-dependence of translation; Pp-luc mRNA with a 7-methyl-guanosine cap was translated greater than ten-fold more efficiently than was the same mRNA with an A(5′)ppp(5′)G cap (Figure

7B). Both RNAs were equally stable in the Drosophila lysate, showing that this difference in efficiency cannot be merely explained by more rapid decay of the mRNA with an adenosine cap (see also Gebauer et al., EMBO J., 18:6146-54 (1999)). Although the translational machinery can discriminate between Pp-luc mRNAs with 7-methyl-guanosine and adenosine caps, the two mRNAs were equally susceptible to RNAi in the presence of Pp-dsRNA (Figure 7C). These results suggest that steps in cap recognition are not involved in RNAi.

dsRNA Is Processed to 21-23 nt Species

RNAs 25 nt in length are generated from both the sense and anti-sense strands of genes undergoing post-transcriptional gene silencing in plants (Hamilton and Baulcombe, Science, 286:950-2 (1999)). Denaturing acrylamide-gel analysis of the products formed in a two-hour incubation of uniformly ^{32}P-radiolabeled dsRNAs and capped asRNA in lysate under standard RNAi conditions, in the presence or absence of target mRNAs. It was found that dsRNA is also processed to small RNA fragments. When incubated in lysate, approximately 15% of the input radioactivity of both the 501 bp Rr-dsRNA and the 505 bp Pp-dsRNA appeared in 21 to 23 nt RNA fragments. Because the dsRNAs are more than 500 bp in length, the 15% yield of fragments implies that multiple 21-23 nt RNAs are produced from each full-length dsRNA molecule. No other stable products were detected. The small RNA species were produced from dsRNAs in which both strands were uniformly ^{32}P-radiolabeled. Formation of the 21-23 nt RNAs from the dsRNA did not require the presence of the corresponding mRNA, demonstrating that the small RNA species is generated by processing of the dsRNA, rather than as a product of dsRNA-targeted mRNA degradation. It was noted that 22 nucleotides corresponds to two turns of an A-form RNA-RNA helix.

When dsRNAs radiolabeled within either the sense or the anti-sense strand were incubated with lysate in a standard RNAi reaction, 21-23 nt RNAs were generated with comparable efficiency. These data support the idea that the 21-23 nt RNAs are

generated by symmetric processing of the dsRNA. A variety of data support the idea that the 21-23 nt RNA is efficiently generated only from dsRNA and is not the consequence of an interaction between single-stranded RNA and the dsRNA. First, a ^{32}P-radiolabeled 505 nt Pp-luc sense RNA or asRNA was not efficiently converted to the 21-23 nt product when it was incubated with 5 nM nonradioactive 505 bp Pp-dsRNA. Second, in the absence of mRNA, a 501 nt 7-methyl-guanosine-capped Rr-asRNA produced only a barely detectable amount of 21-23 nt RNA (capped single-stranded RNAs are as stable in the lysate as dsRNA, Tuschl et al., Genes Dev., 13:3191-7 (1999)), probably due to a small amount of dsRNA contaminating the anti-sense preparation. However, when Rr-luc mRNA was included in the reaction with the ^{32}P-radiolabeled, capped Rr-asRNA, a small amount of 21-23 nt product was generated, corresponding to 4% of the amount of 21-23 nt RNA produced from an equimolar amount of Rr-dsRNA. This result is unlikely to reflect the presence of contaminating dsRNA in the Rr-asRNA preparation, since significantly more product was generated from the asRNA in the presence of the Rr-luc mRNA than in the absence. Instead, the data suggest that asRNA can interact with the complementary mRNA sequences to form dsRNA in the reaction and that the resulting dsRNA is subsequently processed to the small RNA species. Rr-asRNA can support a low level of bona fide RNAi in vitro (see below), consistent with this explanation.

It was next asked if production of the 21-23 nt RNAs from dsRNA required ATP. When the 505 bp Pp-dsRNA was incubated in a lysate depleted for ATP by treatment with hexokinase and glucose, 21-23 nt RNA was produced, albeit 6 times slower than when ATP was regenerated in the depleted lysate by the inclusion of creatine kinase and creatine phosphate. Therefore, ATP may not be required for production of the 21-23 nt RNA species, but may instead simply enhance its formation. Alternatively, ATP may be required for processing of the dsRNA, but at a concentration less than that remaining after hexokinase treatment. The molecular basis for the slower mobility of the small RNA fragments generated in the ATP-depleted lysate is not understood.

Wagner and Sun (Wagner and Sun, Nature, 391:744-745 (1998)) and Sharp (Sharp, Genes Dev., 13:139-41 (1999)) have speculated that the requirement for dsRNA in gene silencing by RNAi reflects the involvement of a dsRNA-specific adenosine deaminase in the process. dsRNA adenosine deaminases unwind dsRNA by converting adenosine to inosine, which does not base-pair with uracil. dsRNA adenosine deaminases function in the post-transcriptional editing of mRNA (for review see Bass, Trends Biochem. Sci., 22:157-62 (1997)). To test for the involvement of dsRNA adenosine deaminase in RNAi, the degree of conversion of adenosine to inosine in the 501 bp Rr-luc and 505 bp Pp-luc dsRNAs after incubation with Drosophila embryo lysate in a standard in vitro RNAi reaction was examined. Adenosine deamination in full-length dsRNA and the 21-23 nt RNA species was assessed by two-dimensional thin-layer chromatography. Inorganic phosphate (P_i,) was produced by the degradation of mononucleotides by phosphatases that contaminate commercially available nuclease P1 (Auxilien et al., J. Mol. Biol., 262:437-458 (1996)). The degree of adenosine deamination in the 21-23 nt species was also determined. The full-length dsRNA radiolabeled with [^{32}P]-adenosine was incubated in the lysate, and both the full-length dsRNA and the 21-23 nt RNA products were purified from a denaturing acrylamide gel, cleaved to mononucleotides with nuclease P1, and analyzed by two-dimensional thin-layer chromatography.

A significant fraction of the adenosines in the full-length dsRNA were converted to inosine after 2 hours (3.1% and 5.6% conversion for Pp-luc and Rr-luc dsRNAs, respectively). In contrast, only 0.4% (Pp-dsRNA) or 0.7% (Rr-dsRNA) of the adenosines in the 21-23 nt species were deaminated. These data imply that fewer than 1 in 27 molecules of the 21-23 nt RNA species contain an inosine. Therefore, it is unlikely that dsRNA-dependent adenosine deamination within the 21-23 nt species is required for its production.

asRNA Generates a Small Amount of RNAi in vitro

When mRNA was ^{32}P-radiolabeled within the 5′-7-methyl-guanosine cap, stable 5′ decay products accumulated during the RNAi reaction. Such stable 5′decay products were observed for both the Pp-luc and Rr-luc mRNAs when they were incubated with their cognate dsRNAs. Previously, it was reported that efficient RNAi does not occur when asRNA is used in place of dsRNA (Tuschl et al., Genes Dev., 13:3191-7 (1999)). Nevertheless, mRNA was measurably less stable when incubated with asRNA than with buffer (Figures 8A and 8B). This was particularly evident for the Rr-luc mRNA: approximately 90% of the RNA remained intact after a 3-hour incubation in lysate, but only 50% when asRNA was added. Less than 5% remained when dsRNA was added. Interestingly, the decrease in mRNA stability caused by asRNA was accompanied by the formation of a small amount of the stable 5′-decay products characteristic of the RNAi reaction with dsRNA. This finding parallels the observation that a small amount of 21-23 nt product formed from the asRNA when it was incubated with the mRNA (see above) and lends strength to the idea that asRNA can enter the RNAi pathway, albeit inefficiently.

mRNA Cleavage Sites Are Determined by the Sequence of the dsRNA

The sites of mRNA cleavage were examined using three different dsRNAs, 'A,' 'B,' and 'C,' displaced along the Rr-luc sequence by approximately 100 nts. Denaturing acrylamide-gel analysis of the stable, 5′-cleavage products produced after incubation of the Rr-luc mRNA for the indicated times with each of the three dsRNAs, 'A,' 'B,' and 'C,' or with buffer (Ø) was performed. The positions of these relative to the Rr-luc mRNA sequence are shown in Figure 9. Each of the three dsRNAs was incubated in a standard RNAi reaction with Rr-luc mRNA ^{32}P-radiolabeled within the 5′-cap. In the absence of dsRNA, no stable 5′-cleavage products were detected for the mRNA, even after 3 hours of incubation in lysate. In contrast, after a 20-minute incubation, each of the three dsRNAs produced a ladder of bands corresponding to a set of mRNA cleavage products characteristic for that particular dsRNA. For each dsRNA, the stable, 5′

mRNA cleavage products were restricted to the region of the Rr-luc mRNA that corresponded to the dsRNA (Figures 9 and 10). For dsRNA 'A,' the lengths of the 5′-cleavage products ranged from 236 to just under ~750 nt; dsRNA 'A' spans nucleotides 233 to 729 of the Rr-luc mRNA. Incubation of the mRNA with dsRNA 'B' produced mRNA 5′-cleavage products ranging in length from 150 to ~600 nt; dsRNA 'B' spans nucleotides 143 to 644 of the mRNA. Finally, dsRNA 'C' produced mRNA cleavage products from 66 to ~500 nt in length. This dsRNA spans nucleotides 50 to 569 of the Rr-luc mRNA. Therefore, the dsRNA not only provides specificity for the RNAi reaction, selecting which mRNA from the total cellular mRNA pool will be degraded, but also determines the precise positions of cleavage along the mRNA sequence.

The mRNA Is Cleaved at 21-23 Nucleotide Intervals

To gain further insight into the mechanism of RNAi, the positions of several mRNA cleavage sites for each of the three dsRNAs were mapped (Figure 10). High resolution denaturing acrylamide-gel analysis of a subset of the 5′-cleavage products described above was performed. Remarkably, most of the cleavages occurred at 21-23 nt intervals (Figure 10). This spacing is especially striking in light of our observation that the dsRNA is processed to a 21-23 nt RNA species and the finding of Hamilton and Baulcombe that a 25 nt RNA correlates with post-transcriptional gene silencing in plants (Hamilton and Baulcombe, Science, 286:950-2 (1999)). Of the 16 cleavage sites we mapped (2 for dsRNA 'A,' 5 for dsRNA 'B,' and 9 for dsRNA 'C'), all but two reflect the 21-23 nt interval. One of the two exceptional cleavages was a weak cleavage site produced by dsRNA 'C' (indicated by an open blue circle in Figure 10). This cleavage occurred 32 nt 5′ to the next cleavage site. The other exception is particularly intriguing. After four cleavages spaced 21-23 nt apart, dsRNA 'C' caused cleavage of the mRNA just nine nt 3′ to the previous cleavage site (red arrowhead in Figure 10). This cleavage occurred in a run of seven uracil residues and appears to "reset" the ruler for cleavage; the next cleavage site was 21-23 nt 3′ to the exceptional site. The three subsequent cleavage sites that we mapped were also spaced 21-23 nt apart. Curiously,

of the sixteen cleavage sites caused by the three different dsRNAs, fourteen occur at uracil residues. The significance of this finding is not understood, but it suggests that mRNA cleavage is determined by a process which measures 21-23 nt intervals and which has a sequence preference for cleavage at uracil. Results show that the 21-23 nt RNA species produced by incubation of ~500 bp dsRNA in the lysate caused sequence-specific interference in vitro when isolated from an acrylamide gel and added to a new RNAi reaction in place of the full-length dsRNA.

A Model for dsRNA-directed mRNA Cleavage

Without wishing to be bound by theory, the biochemical data described herein, together with recent genetic experiments in C. elegans and Neurospora (Cogoni and Macino, Nature, 399:166-9 (1999); Grishok et al., Science, 287: 2494-7 (2000); Ketting et al., Cell, 99:133-41 (1999); Tabara et al., Cell, 99:123-32 (1999)), suggest a model for how dsRNA targets mRNA for destruction (Figure 11). In this model, the dsRNA is first cleaved to 21-23 nt long fragments in a process likely to involve genes such as the C. elegans loci rde-1 and rde-4. The resulting fragments, probably as short asRNAs bound by RNAi-specific proteins, would then pair with the mRNA and recruit a nuclease that cleaves the mRNA. Alternatively, strand exchange could occur in a protein-RNA complex that transiently holds a 21-23 nt dsRNA fragment close to the mRNA. Separation of the two strands of the dsRNA following fragmentation might be assisted by an ATP-dependent RNA helicase, explaining the observed ATP enhancement of 21-23 nt RNA production.

It is likely that each small RNA fragment produces one, or at most two, cleavages in the mRNA, perhaps at the 5′ or 3′ ends of the 21-23 nt fragment. The small RNAs may be amplified by an RNA-directed RNA polymerase such as that encoded by the ego-1 gene in C. elegans (Smardon et al., Current Biology, 10:169-178 (2000)) or the qde-1 gene in Neurospora (Cogoni and Macino, Nature, 399:166-9 (1999)), producing long-lasting post-transcriptional gene silencing in the absence of the dsRNA that initiated the RNAi effect. Heritable RNAi in C. elegans requires the rde-1

and rde-4 genes to initiate, but not to persist in subsequent generations. The rde-2, rde-3, and mut-7 genes in C. elegans are required in the tissue where RNAi occurs, but are not required for initiation of heritable RNAi (Grishok et al., Science, in press 2000). These 'effector' genes (Grishok et al., Science, in press 2000) are likely to encode proteins functioning in the actual selection of mRNA targets and in their subsequent cleavage. ATP may be required at any of a number of steps during RNAi, including complex formation on the dsRNA, strand dissociation during or after dsRNA cleavage, pairing of the 21-23 nt RNAs with the target mRNA, mRNA cleavage, and recycling of the targeting complex. Testing these ideas with the in vitro RNAi system will be an important challenge for the future. Some genes involved in RNAi are also important for transposon silencing and co-suppresion. Co-suppression is a broad biological phenomenon spanning plants, insects and perhaps humans. The most likely mechanism in Drosophila melanogaster is transcriptional silencing (Pal-Bhanra et al, Cell 99: 35-36. Thus, 21-23 nt fragments are likely to be involved in transcriptional control, as well as in post-transcriptional cotrol.

Example 3 Isolated 21-23 mers caused sequence-specific interference when added to a new RNAi reaction

Isolation of 21-23 nt fragments from incubation reaction of 500 bp dsRNA in lysate.

Double-stranded RNA (500 bp from) was incubated at 10 nM concentration in Drosophila embryo lysate for 3 h at 25° C under standard conditions as described herein. After deproteinization of the sample, the 21-23 nt reaction products were separated from unprocessed dsRNA by denaturing polyacrylamide (15%) gel electrophoresis. For detection of the non-radiolabeled 21-23 nt fragments, an incubation reaction with radiolabeled dsRNA was loaded in a separate lane of the same gel. Gel slices containing the non-radioactive 21-23 nt fragments were cut out and the 21-23 nt fragments were eluted from the gel slices at 4° C overnight in 0.4 ml 0.3 M NaCl. The RNA was recovered from the supernatant by ethanol precipitation and centrifugation. The RNA pellet was dissolved in 10 µl of lysis buffer. As control, gel slices slightly above and

below the 21-23 nt band were also cut out and subjected to the same elution and precipitation procedures. Also, a non-incubated dsRNA loaded on the 15% gel and a gel slice corresponding to 21-23 nt fragments was cut out and eluted. All pellets from the control experiments were dissolved in 10 µl lysis buffer. The losses of RNA during recovery from gel slices by elution are approx. 50%.

Incubation of purified 21-23 nt fragments in a translation-based RNAi assay

1 µl of the eluted 21-23 mer or control RNA solution was used for a standard 10 µl RNAi incubation reaction (see above). The 21-23 mers were preincubated in the lysate containing reaction mixture for 10 or 30 min before the addition of the target and control mRNA. During pre-incubation, proteins involved in RNA interference may re-associate with the 21-23 mers due to a specific signal present on these RNAs. The incubation was continued for another hour to allow translation of the target and control mRNAs. The reaction was quenched by the addition of passive lysis buffer (Promega), and luciferase activity was measured. The RNA interference is the expressed as the ratio of target to control luciferase activity normalized by an RNA-free buffer control. Specific suppression of the target gene was observed with either 10 or 30 minutes pre-incubation. The suppression was reproducible and reduced the relative ratio of target to control by 2-3 fold. None of the RNA fragments isolated as controls showed specific interference. For comparison, incubation of 5 nM 500 bp dsRNA (10 min pre-incubation) affects the relative ratio of control to target gene approx. 30-fold.

Stability of isolated 21-23 nt fragments in a new lysate incubation reaction.

Consistent with the observation of RNAi mediated by purified 21-23 nt RNA fragment, it was found that 35% of the input 21-23 nt RNA persists for more than 3 h in such an incubation reaction. This suggests that cellular factors associate with the deproteinized 21-23 nt fragments and reconstitute a functional mRNA-degrading particle. Signals connected with these 21-23 nt fragments, or their possible double stranded nature or specific lengths are likely responsible for this observation. The 21-23

nt fragments have a terminal 3' hydroxyl group, as evidenced by altered mobility on a sequencing gel following periodate treatment and beta-elimination.

Example 4 21-23-mers purified by non-denaturing methods caused sequence-specific interference when added to a new RNAi reaction.

Fifty nanomolar double-stranded RNA (501 bp Rr-luc dsRNA, as described in example 1) was incubated in a 1 ml in vitro reaction with lysate at 25°C (see example 1). The reaction was then stopped by the addition of an equal volume of 2x PK buffer (see example 1) and proteinase K was added to a final concentration of 1.8 μg/μl. The reaction was incubated for an additional 1 h at 25°C, phenol extracted, and then the RNAs were precipitated with 3 volumes of ethanol. The ethanol precipitate was collected by centrifugation, and the pellet was resuspended in 100 μl of lysis buffer and applied to a Superdex HR 200 10/30 gel filtration column (Pharmacia) run in lysis buffer at 0.75 ml/min. 200 μl fractions were collected from the column. Twenty μl of 3 M sodium acetate and 20 μg glycogen was added to each fraction, and the RNA was recovered by precipitation with 3 volumes of ethanol. The precipitates were resuspended in 30 μl of lysis buffer. Column profiles following the fractionation of 32P-labeled input RNA are shown in Figure 13A.

One microliter of each resuspended fraction was tested in a 10 μl standard in vitro RNAi reaction (see example 1). This procedure yields a concentration of RNA in the in vitro RNAi reaction that is approximately equal to the concentration of that RNA species in the original reaction prior to loading on the column. The fractions were preincubated in the lysate containing reaction mixture for 30 min before the addition of 10 nM Rr-luc mRNA target and 10 nM Pp-luc control mRNA. During pre-incubation, proteins involved in RNA interference may re-associate with the 21-23-mers due to a specific signal present on these RNAs. The incubation was continued for another three hours to allow translation of the target and control mRNAs. The reaction was quenched by the addition of passive lysis buffer (Promega), and luciferase activity was measured. The suppression of Rr-luc mRNA target expression by the purified 21-23 nt fragments

was reproducible and reduced the relative ratio of target to control by >30-fold, an amount comparable to a 50 nM 500 bp dsRNA control. Suppression of target mRNA expression was specific: little or no effect on the expression of the Pp-luc mRNA control was observed.

The data show that the both the fractions containing uncleaved dsRNA (fractions 3 - 5) or long, partially cleaved dsRNA (fractions 7 - 13) and the fractions containing the fully processed 21-23 nt siRNAs (fractions 41 - 50) mediate effective RNA interference in vitro (Figure 13B). Suppression of target mRNA expression was specific: little or no effect on the expression of the Pp-luc mRNA control was observed (Figure 13C). These data, together with those in the earlier examples, demonstrate that the 21-23 nt siRNAs are (1) true intermediates in the RNAi pathway and (2) effective mediators of RNA interference in vitro.

Example 5 21-nucleotide siRNA duplexes mediate RNA interference in human tissue cultures

Methods

RNA preparation

21 nt RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified (Elbashir, S.M., Lendeckel, W. & Tuschl, T., *Genes & Dev. 15*, 188-200 (2001)), followed by Sep-Pak C18 cartridge (Waters, Milford, MA, USA) purification (Tuschl, t., *et al., Biochemistry, 32:*11658-11668 (1993)). The siRNA sequences targeting GL2 (Acc. X65324) and GL3 luciferase (Acc. U47296) corresponded to the coding regions 153-173 relative to the first nucleotide of the start codon, siRNAs targeting RL (Acc. AF025846) corresponded to region 119-129 after the start codon. Longer RNAs were transcribed with T7 RNA polymerase from PCR products, followed by gel and Sep-Pak purification. The 49 and 484 bp GL2 or GL3 dsRNAs corresponded to position 113-161 and 113-596, respectively, relative to the start of translation; the 50 and 501 bp RL dsRNAs corresponded to position 118-167

and 118-618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (Kehlenbach, R.H., *et al., J. Cell Biol., 141:*863-874 (1998)), whereby 50 and 501 bp hG dsRNA corresponded to position 118-167 and 118-618, respectively, to the start codon.

For annealing of siRNAs, 20 μM single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h at 37 °C. The 37 °C incubation step was extended overnight for the 50 and 500 bp dsRNAs, and these annealing reactions were performed at 8.4 μM and 0.84 μM strand concentrations, respectively.

Cell culture

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 25 °C. 293, NIH/3T3, HeLa S3, COS-7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were regularly passaged to maintain exponential growth. 24 h before transfection at approx. 80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics ($1\text{-}3 \times 10^5$ cells/ml) and transferred to 24-well plates (500 μl/well). S2 cells were not trypsinized before splitting. Transfection was carried out with Lipofectamine 2000 reagent (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 μg pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 μg pRL-TK (Promega), and 0.28 μg siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 μl per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cell lines after co-transfection of 1.1 μg hGFP-encoding pAD3[22] and 0.28 μg invGL2 siRNA, and were 70-90%. Reporter plasmids were

amplified in XL-1 Blue (Strategene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

Results

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) homologous in sequence to the silenced gene (Fire, A., *Trends Genet., 15:*358-363 (1999); Sharp, P.A. & Zamore, P.D., *Science, 287:*2431-2433 (2000); Sijen, T. & Kooter, J.M., *Bioessays, 22:*520-531 (2000); Bass, B.L., *Cell, 101:*235-238 (2000); Hammond, S.M., *et al., Nat. Rev. Genet., 2:*110-119 (2001)). The mediators of sequence-specific mRNA degradation are 21 and 22 nt small interfering RNAs (siRNAs) generated by RNase III cleavage from longer dsRNAs[6-10] (Hamilton, A.J. &Baulcombe, D.C., *Science, 286:*950-952 (1999); Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Zamore, P.D., *et al., Cell, 101:*25-33 (2000); Bernstein, E., *et al., Naature, 409:*363-366 (2001); Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). As shown herein, 21 nt siRNA duplexes are able to specifically suppress reporter gene expression in multiple mammalian tissue cultures, including human embryonic kidney (293) and HeLa cells. In contrast to 50 or 500 bp dsRNAs, siRNAs do not activate the interferon response. These results indicate that siRNA duplexes are a general tool for sequence-specific inactivation of gene function in mammalian cells.

Base-paired 21 and 22 nt siRNAs with overhanging 3' ends mediate efficient sequence-specific mRNA degradation in lysates prepared from *D. melanogaster* embryos (Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). To test whether siRNAs are also capable of mediating RNAi in tissue culture, 21 nt siRNA duplexes with symmetric 2 nt 3' overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases (Figures 14A, 14B) were constructed. The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL, into *D. melanogaster Schneider S2* cells or mammalian cells using cationic liposomes.

Luciferase activities were determined 20 h after transfection. In all cell lines tested, specific reduction of the expression of the reporter genes in the presence of cognate siRNA duplexes was observed (Figures 15A-15J). Remarkably, the absolute luciferase expression levels were unaffected by non-cognate siRNAs, indicating the absence of harmful side effects by 21 nt RNA duplexes (e.g. Figures 16A-16D, for HeLa cells). In *D. melanogaster* S2 cells (Figures 15A, 15B), the specific inhibition of luciferases was complete, and similar to results previously obtained for longer dsRNAs (Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Caplen, N.J., *et al., Gene, 252:*95-105 (2000); Clemens, M & Williams, B., *Cell, 13:*565-572 (1978); Ui-Tei, K., *et al., FEBS Letters, 479:*79-82 (2000)). In mammalian cells, where the reporter genes were 50- to 100-fold stronger expressed, the specific suppression was less complete (Figures 15C-15J). GL2 expression was reduced 3- to 12-fold, GL3 expression 9- to 25-fold, and RL expression 1- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (Figures 15I, 15J). It is likely that the lack of reduction of RL expression in 293 cells is due to its 5- to 20-fold higher expression compared to any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

The 2 nt 3' overhang in all siRNA duplexes, except for uGL2, was composed of (2'-deoxy) thymidine. Substitution of uridine by thymidine in the 3' overhang was well tolerated in the *D. melanogaster* in vitro system, and the sequence of the overhang was uncritical for target recognition (Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). The thymidine overhang was chosen, because it is supposed to enhance nuclease resistance of siRNAs in the tissue culture medium and within transfected cells. Indeed, the thymidine-modified GL2 siRNA was slightly more potent than the unmodified uGL2 siRNA in all cell lines tested (Figures 15A, 15C, 15E, 15G, 15I). It is

conceivable that further modifications of the 3' overhanging nucleotides will provide additional benefits to the delivery and stability of siRNA duplexes.

In co-transfection experiments, 25 nM siRNA duplexes with respect to the final volume of tissue culture medium were used (Figures 15A-15J, 16A-16F). Increasing the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies due to competition for liposome encapsulation between plasmid DNA and siRNA. Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect, even though the siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing, and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene targeting experiments.

In order to monitor the effect of longer dsRNAs on mammalian cells, 50 and 500 bp dsRNAs cognate to the reporter genes were prepared. As non-specific control, dsRNAs from humanized GFP (hG) (Kehlenbach, R.H., *et al., J. Cell Biol., 141:*863-874 (1998)) was used. When dsRNAs were co-transfected, in identical amounts (not concentrations) to the siRNA duplexes, the reporter gene expression was strongly and unspecifically reduced. This effect is illustrated for HeLa cells as a representative example (Figures 16A-16D). The absolute luciferase activities were decreased unspecifically 10- to 20-fold by 50 bp dsRNA, and 20- to 200-fold by 500 bp dsRNA co-transfection, respectively. Similar unspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold unspecific reduction was observed only for 500 bp dsRNAs. Unspecific reduction in reporter gene expression by dsRNA > 30 bp was expected as part of the interferon response (Matthews, M., Interactions between viruses and the cellular machinery for protein synthesis in *Translational Control* (eds., Hershey, J., Matthews,M. & Sonenberg, N.) 505-548 (Cold Spring Harbor Laboratory Press, Plainview, NY; 1996); Kumar, M. & Carmichael, G.G., *Microbiol. Mol. Biol. Rev., 62:*1415-1434 (1998); Stark, G.R., *et al., Annu. Rev. Biochem., 67:*227-264 (1998)). Surprisingly, despite the strong unspecific decrease in reporter gene expression,

additional sequence-specific, dsRNA-mediated silencing were reproducibly detected. The specific silencing effects, however, were only apparent when the relative reporter gene activities were normalized to the hG dsRNA controls (Figures 16E, 16F). A 2- to 10-fold specific reduction in response to cognate dsRNA was observed, also in the other three mammalian cell lines tested. Specific silencing effects with dsRNAs (356-1662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments (Ui-Tei, K., *et al., FEBS Letters, 479:*79-82 (2000)). Also, CHO-K1 cells appear to be deficient in the interferon response. In another report, 293, NIH/3T3, and BHK-21 cells were tested for RNAi using luciferase/lacZ reporter combinations and 829 bp specific lacZ or 717 bp unspecific GFP dsRNA (Caplen, N.J., *et al., Gene, 252:*95-105 (2000)). The failure of detecting RNAi in this case is likely due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, the results described herein indicate that RNAi is active in mammalian cells, but that the silencing effect is difficult to detect if the interferon system is activated by dsRNA >30 bp.

The mechanism of the 21 nt siRNA-mediated interference process in mammalian cells remains to be uncovered, and silencing may occur post-transcriptional and/or transcriptional. In *D. melanogaster* lysate, siRNA duplexes mediate post-transcriptional gene silencing by reconstitution of a siRNA-protein complexes (siRNPs), which are guiding mRNA recognition and targeted cleavage (Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Zamore, P.D., *et al., Cell, 101:*25-33 (2000); Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). In plants, dsRNA-mediated post-transcriptional silencing has also been linked to RNA-directed DNA methylation, which may also be directed by 21 nt siRNAs (Wassenegger, M., *Plant Mol. Biol, 43:*203-220 (2000); Finnegan, E.J., *et al., Curr. Biol., 11:*R99-R102 (2000)). Methylation of promoter regions can lead to transcriptional silencing (Metter, M.F., *et al., EMBO J., 19:*5194-5201 (2000)), but methylation in coding sequences must not (Wang, M.-B., *RNA, 7:*16-28 (2001)). DNA methylation and transcriptional silencing in mammals are

well-documented processes (Kass, S.U., *et al., Trends Genet., 13:*444-449 (1997); Razin, A., *EMBO J, 17:*4905-4908 (1998)), yet they have not been linked to post-transcriptional silencing. Methylation in mammals is predominantly directed towards CpG residues. Because there is no CpG in the RL siRNA, but RL siRNA mediates specific silencing in mammalian tissue culture, it is unlikely that DNA methylation is critical for our observed silencing process. In summary, described herein, is siRNA-mediated gene silencing in mammalian cells. The use of 21 nt siRNAs holds great promise for inactivation of gene function in human tissue culture and the development of gene-specific therapeutics.

While this invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims

CLAIMS

What is claimed is:

1. Isolated RNA of from about 21 to about 23 nucleotides that mediates RNA interference of an mRNA to which it corresponds.

2. Isolated RNA of claim 1 that comprises a terminal 3' hydroxyl group.

3. Isolated RNA of claim 1 which is chemically synthesized RNA or an analog of a naturally occurring RNA.

4. An analog of isolated RNA of claim 1, wherein the analog differs from the RNA of claim 1 by the addition, deletion, substitution or alteration of one or more nucleotides.

5. Isolated RNA of from about 21 to about 23 nucleotides that inactivates a corresponding gene by transcriptional silencing.

6. A soluble extract that mediates RNA interference.

7. The soluble extract of Claim 6, wherein the extract is derived from Drosophila embryos.

8. The soluble extract of Claim 7 wherein the extract is derived from syncytial blastoderm Drosophila embryos.

9. A method of producing RNA of from about 21 to about 23 nucleotides in length comprising:

(a) combining double-stranded RNA with a soluble extract that mediates RNA interference, thereby producing a combination; and

(b) maintaining the combination of a) under conditions in which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides in length.

10. The method of Claim 9, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

11. The method of Claim 9 further comprising isolating the RNA of from about 21 to about 23 nucleotides from the combination.

12. RNA of about 21 to about 23 nucleotides produced by the method of Claim 9.

13. A method of producing RNA of from about 21 to about 23 nucleotides in length that mediates RNA interference of mRNA of a gene to be degraded, comprising:

(a) combining double-stranded RNA that corresponds to a sequence of the gene to be degraded with a soluble extract that mediates RNA interference, thereby producing a combination; and

(b) maintaining the combination of (a) under conditions under which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides that mediates RNA interference of the mRNA of the gene to be degraded, thereby producing RNA of from about 21 to about 23 nucleotides that mediates RNA interference of the mRNA.

14. The method of Claim 13, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

15. The method of Claim 13 further comprising isolating RNA of from about 21 to about 23 nucleotides from the combination.

16. Isolated RNA of from about 21 to about 23 nucleotides produced by the method of Claim 15.

17. A method of mediating RNA interference of mRNA of a gene in a cell or organism comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into the cell or organism;

(b) maintaining the cell or organism produced in (a) under conditions under which degradation of the mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism.

18. The method of Claim17 wherein the RNA of (a) is a chemically synthesized RNA or an analog of naturally occurring RNA.

19. The method of Claim 17, wherein the gene encodes a cellular mRNA or a viral mRNA.

20. A method of mediating RNA interference of mRNA of a gene in a cell or organism in which RNA interference occurs, comprising:

(a) combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract that mediates RNA interference, thereby producing a combination;

(b) maintaining the combination produced in (a) under conditions under which the double- stranded RNA is processed to RNA of from about 21 to about 23 nucleotides, thereby producing RNA of from about 21 to about 23 nucleotides;

(c) isolating RNA of from about 21 to about 23 nucleotides produced in (b);

(d) introducing RNA isolated in (c) into the cell or organism; and

(e) maintaining the cell or organism produced in (d) under conditions under which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism.

21. The method of Claim 20, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

22. The method of Claim 20, wherein the RNA is isolated using gel electrophoresis.

23. A method of mediating RNA interference of mRNA of a gene in a cell or organism in which RNA interference occurs, comprising: (a) introducing into the cell or organism RNA of from about 21 to about 23 nucleotides that mediates RNA interference of mRNA of the gene, thereby producing a cell or organism that contains the RNA and (b) maintaining the cell or organism that contains the RNA under conditions under which RNA interference occurs, thereby mediating RNA interference of mRNA of the gene in the cell or organism.

24. The method of claim 23, wherein the RNA of from about 21 to about 23 nucleotides is chemically synthesized RNA or an analog of RNA that mediates RNA interference.

25. The method of Claim 23, wherein the gene encodes a cellular mRNA or a viral mRNA.

26. A knockdown cell or organism generated by the method of claim 23.

27. The knockdown cell or organism of claim 26, wherein the cell or organism mimics a disease.

28. A method of examining the function of a gene in a cell or organism comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides that targets mRNA of the gene for degradation into the cell or organism, thereby producing a test cell or test organism;

(b) maintaining the test cell or test organism under conditions under which degradation of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is degraded; and

(c) observing the phenotype of the test cell or test organism produced in (b) and, optionally, comparing the phenotype observed to that of an appropriate control cell or control organism, thereby providing information about the function of the gene.

29. The method of Claim 28 wherein the RNA introduced in (a) is chemically synthesized or an analog of RNA that mediates RNA interference.

30. A method of examining the function of a gene in a cell or organism comprising

(a) combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract that mediates RNA interference, thereby producing a combination;

(b) maintaining the combination produced in (a) under conditions under which the double- stranded RNA is processed to RNA of about 21 to about 23 nucleotides, whereby RNA of about 21 to about 23 nucleotides is produced;

(c) isolating RNA of about 21 to about 23 nucleotides produced in (b);

(d) introducing the RNA isolated in (c) into the cell or organism, thereby producing a test cell or test organism;

(e) maintaining the test cell or test organism under conditions under which degradation of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is degraded; and

(f) observing the phenotype of the test cell or test organism produced in (e) and, optionally, comparing the phenotype observed to that of an appropriate control, thereby providing information about the function of the gene.

31. The method of claim 30, wherein the RNA comprises a terminal 3' hydroxyl group.

32. The method of claim 30, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

33. The method of claim 30, wherein the RNA is isolated using gel electrophoresis.

34. A composition comprising biochemical components of a Drosophila cell that process dsRNA to RNA of about 21 to about 23 nucleotides and a suitable carrier.

35. A composition comprising biochemical components of a cell that target mRNA of a gene to be degraded by RNA of about 21 to about 23 nucleotides.

36. A method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNA of from about 21 to about 23 nucleotides that targets the mRNA of the protein for degradation.

37. The method of claim 36 wherein RNA of from about 21 to about 23 nucleotides is chemically synthesized or an analog of RNA that mediates RNA interference.

38. A method of assessing whether an agent acts on a gene product comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into a cell or organism;

(b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs,

(c) introducing the agent into the cell or organism of (b); and

(d) determining whether the agent has an effect on the cell or organism, wherein if the agent has no effect on the cell or organism then the agent acts on the gene product or on a biological pathway that involves the gene product.

39. The method of claim 38, wherein the RNA of from about 21 to about 23 nucleotides is chemically synthesized or an analog of RNA that mediates RNA interference.

40. A method of assessing whether a gene product is a suitable target for drug discovery comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into a cell or organism;

(b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs resulting in decreased expression of the gene; and

(c) determining the effect of the decreased expression of the gene on the cell or organism, wherein if decreased expression has an effect, then the gene product is a target for drug discovery.

41. The method of claim 40, wherein the RNA of from about 21 to about 23 nucleotides is synthetic RNA or an analog of RNA that mediates RNA interference.

42. A gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

43. A pharmaceutical composition comprising RNA of from about 21 to about 23 nucleotides that mediates RNA interference and an appropriate carrier.

44. A method of producing knockdown cells, comprising introducing into cells in which a gene is to be knocked down RNA of about 21 to about 23 nt that targets the mRNA corresponding to the gene and maintaining the resulting cells under conditions under which RNAi occurs, resulting in degradation of the mRNA of the gene, thereby producing knockdown cells.

45. The method of claim 44, wherein the RNA of about 21 to about 23 nucleotides is synthetic RNA or an analog of RNA that mediates RNA interference.

46. A method of identifying target sites within mRNA that are efficiently cleaved by the RNAi process, comprising combining dsRNA corresponding to a sequence of a gene to be degraded, labeled mRNA corresponding to the gene and a soluble extract that mediates RNA interference, thereby producing a combination; maintaining the combination under conditions under which the dsRNA is degraded and identifying sites in the mRNA that are efficiently cleaved.

47. A method of identifying 21-23 nt RNAs that efficiently mediate RNAi, wherein said 21-23 nt RNAs span the target sites identified within the mRNA by the method of claim 46.

48. RNA of claim 16, isolated using gel electrophoresis.

49. RNA of claim 16, isolated using non-denaturing methods.

50. RNA of claim 16, isolated using non-denaturing column chromatography.

RNA Sequence Specific Mediators of RNA Interference

ABSTRACT OF THE DISCLOSURE

The present invention relates to a Drosophila in vitro system which was used to demonstrate that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length. Furthermore, when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of long dsRNA. Thus, these 21-23 nt fragments are the sequence-specific mediators of RNA degradation. A molecular signal, which may be their specific length, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells, where the use of long dsRNAs to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. This specific targeting of a particular gene function is useful in functional genomic and therapeutic applications.

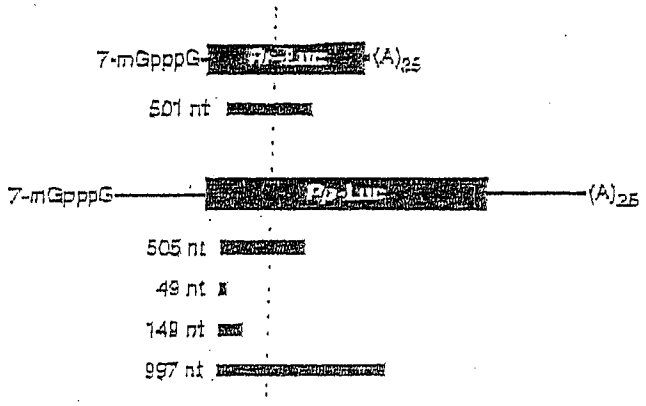


Figure 1

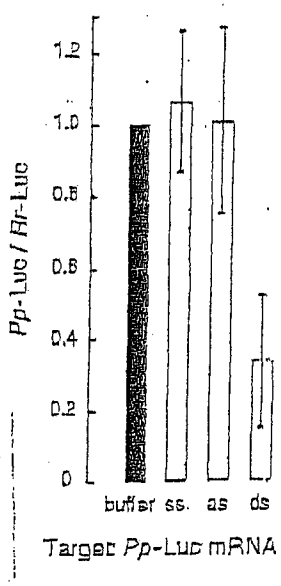


Figure 2A

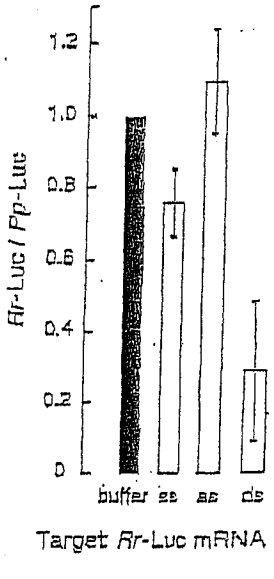


Figure 2B

Docket No.: 0399.2008-002
Title: RNA SEQUENCE-SPECIFIC MEDIATORS...
Inventors: Thomas Tuschl, *et al.*

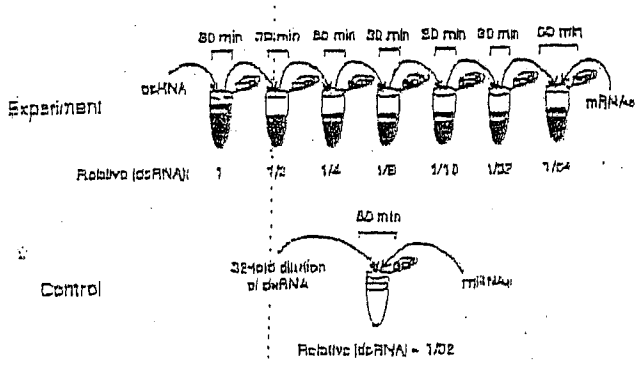


Figure 3A

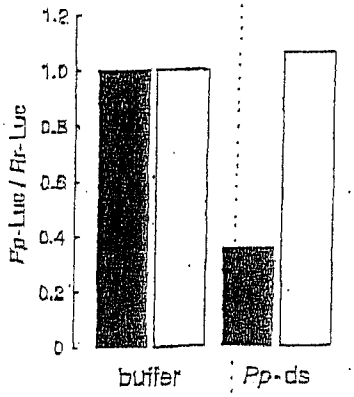


Figure 3B

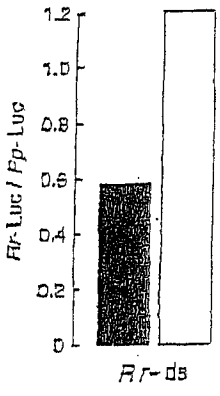


Figure 3C

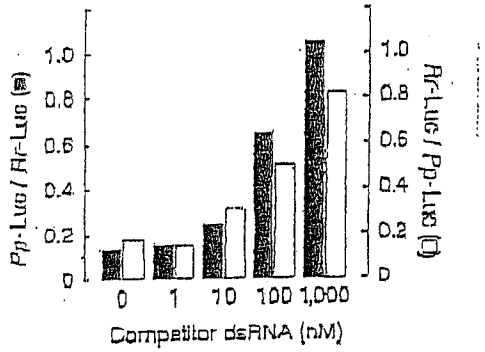


Figure 4

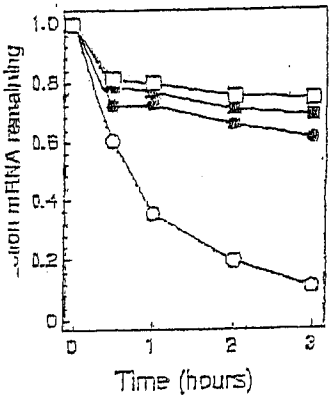


Figure 5A

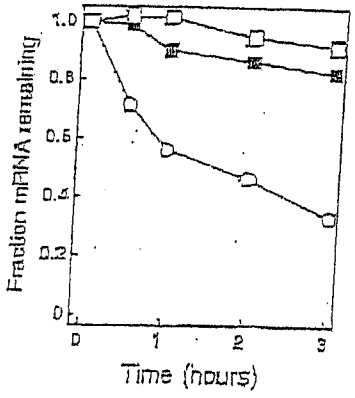


Figure 5B

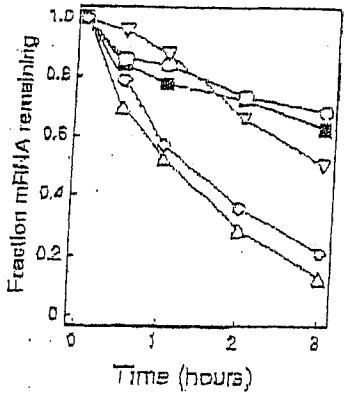


Figure 5C

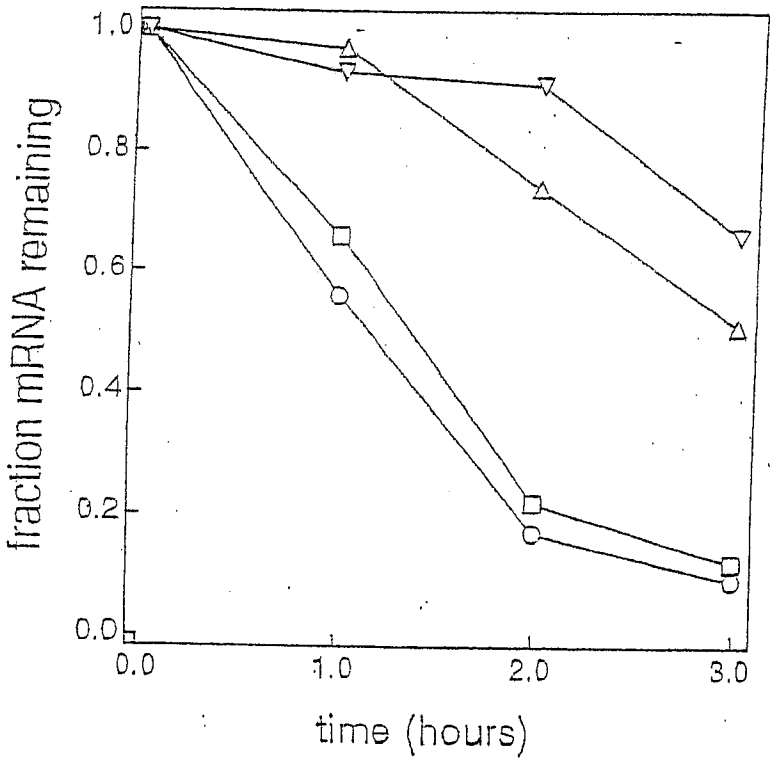


Figure 6

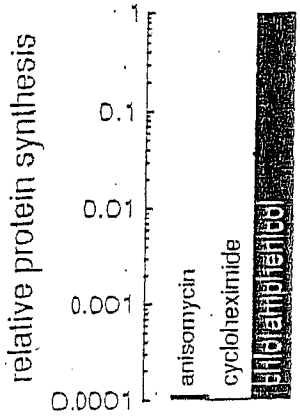


Figure 7A

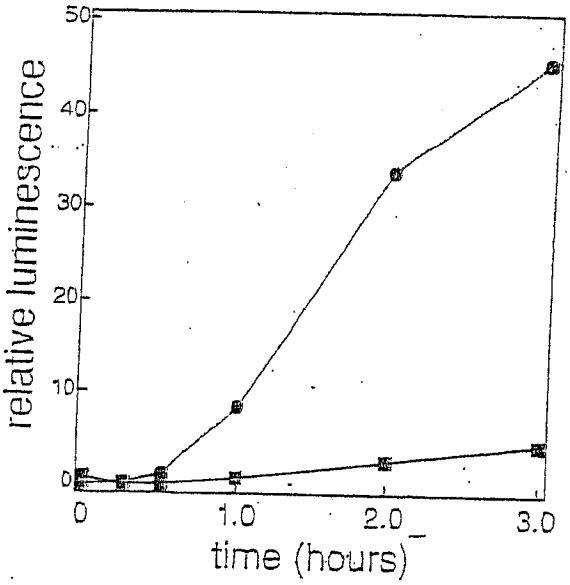


Figure 7B

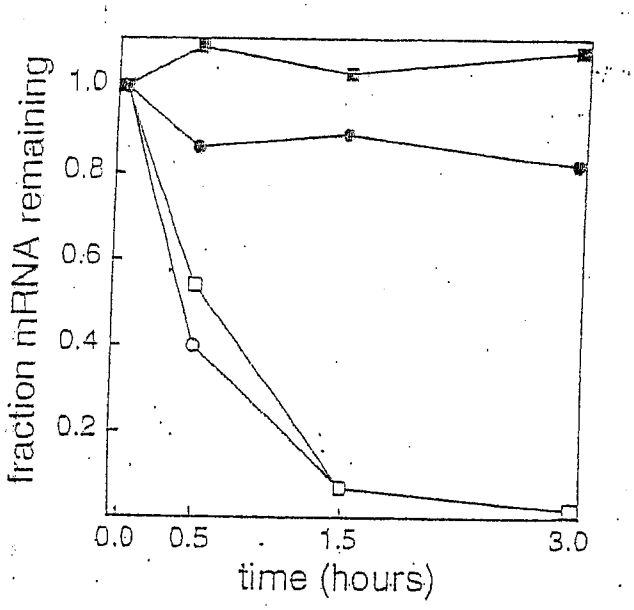


Figure 7C

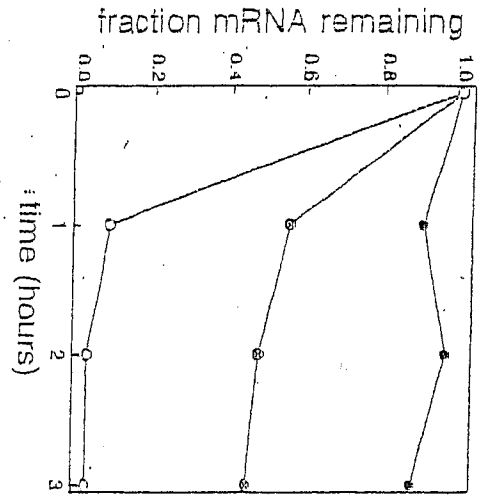


Figure 8A

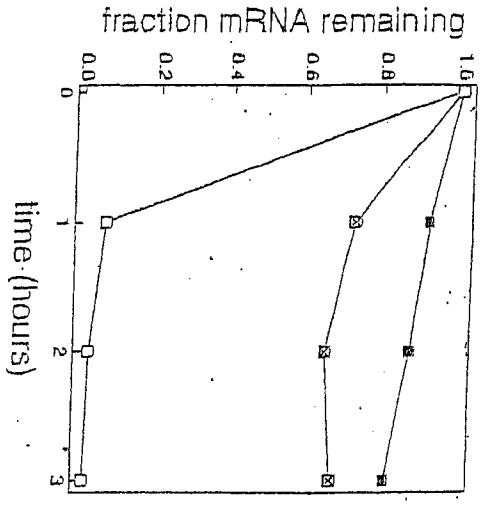


Figure 8B

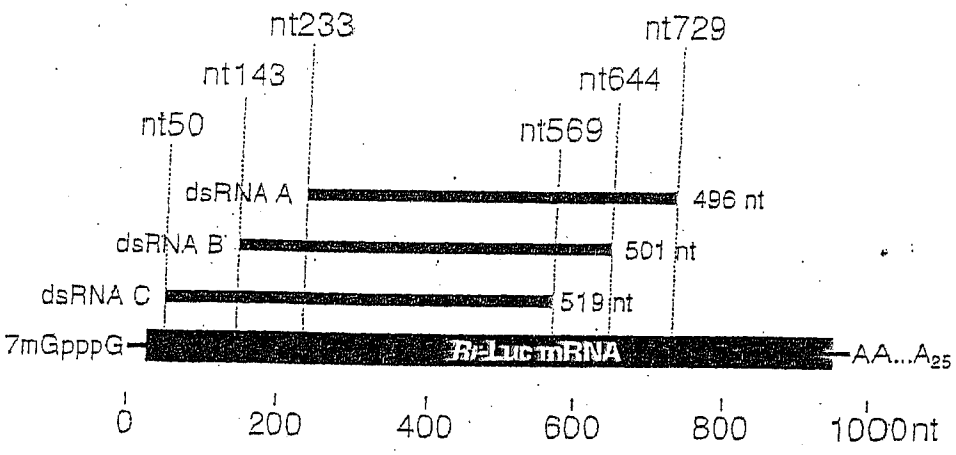


Figure 9

EXHIBIT 5

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WO 01/75164 A2

(54) Title: RNA SEQUENCE-SPECIFIC MEDIATORS OF RNA INTERFERENCE

(57) Abstract: The present invention relates to a Drosophila in vitro system which was used to demonstrate that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length. Furthermore, when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of long dsRNA. Thus , these 21-23 nt fragments are the sequence-specific mediators of RNA degradation. A molecular signal, which may be their specific length, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells, where the use of long dsRNAs to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. This specific targeting of a particular gene function is useful in functional genomic and therapeutic applications.

RNA Sequence-Specific Mediators of RNA Interference

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/265,232, filed January 31, 2001 and U.S. Provisional Application No. 60/193,594, filed March 30, 2000, and claims priority under 35 U.S.C. §119 to European Application No. 00 126 325.0 filed December 1, 2000. The entire teachings of the above applications are incorporated herein by reference.

GOVERNMENT SUPPORT

Work described herein was funded in part by grants from the National Institutes of Health through a United States Public Health Service MERIT award (Grant No. RO1-GM34277) from the National Institutes of Health. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (Fire et al. (1998) Nature 391, 806-811). dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. RNAi involves mRNA degradation, but many of the biochemical mechanisms underlying this interference are unknown. The recapitulation of the essential features of RNAi in vitro is needed for a biochemical analysis of the phenomenon.

SUMMARY OF THE INVENTION

Described herein is gene-specific, dsRNA-mediated interference in a cell-free system derived from syncytial blastoderm Drosophila embryos. The in vitro system complements genetic approaches to dissecting the molecular basis of

RNAi. As described herein, the molecular mechanisms underlying RNAi were examined using the Drosophila in vitro system. Results showed that RNAi is ATP-dependent yet uncoupled from mRNA translation. That is, protein synthesis is not required for RNAi in vitro. In the RNAi reaction, both strands (sense and antisense) of the dsRNA are processed to small RNA fragments or segments of from about 21 to about 23 nucleotides (nt) in length (RNAs with mobility in sequencing gels that correspond to markers that are 21-23 nt in length, optionally referred to as 21-23 nt RNA). Processing of the dsRNA to the small RNA fragments does not require the targeted mRNA, which demonstrates that the small RNA species is generated by processing of the dsRNA and not as a product of dsRNA-targeted mRNA degradation. The mRNA is cleaved only within the region of identity with the dsRNA. Cleavage occurs at sites 21-23 nucleotides apart, the same interval observed for the dsRNA itself, suggesting that the 21-23 nucleotide fragments from the dsRNA are guiding mRNA cleavage. That purified 21-23 nt RNAs mediate RNAi confirms that these fragments are guiding mRNA cleavage.

Accordingly, the present invention relates to isolated RNA molecules (double- stranded; single-stranded) of from about 21 to about 23 nucleotides which mediate RNAi. That is, the isolated RNAs of the present invention mediate degradation of mRNA of a gene to which the mRNA corresponds (mediate degradation of mRNA that is the transcriptional product of the gene, which is also referred to as a target gene). For convenience, such mRNA is also referred to herein as mRNA to be degraded. As used herein, the terms RNA, RNA molecule(s), RNA segment(s) and RNA fragment(s) are used interchangeably to refer to RNA that mediates RNA interference. These terms include double-stranded RNA, single-stranded RNA, isolated RNA (partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA), as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the 21-23 nt RNA or internally (at one or more nucleotides of the RNA). Nucleotides in the RNA molecules of the present invention can also comprise non-standard nucleotides, including

non-naturally occurring nucleotides or deoxyribonucleotides. Collectively, all such altered RNAs are referred to as analogs or analogs of naturally-occurring RNA. RNA of 21-23 nucleotides of the present invention need only be sufficiently similar to natural RNA that it has the ability to mediate (mediates) RNAi. As used herein the phrase "mediates RNAi" refers to (indicates) the ability to distinguish which RNAs are to be degraded by the RNAi machinery or process. RNA that mediates RNAi interacts with the RNAi machinery such that it directs the machinery to degrade particular mRNAs. In one embodiment, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that direct cleavage of specific mRNA to which their sequence corresponds. It is not necessary that there be perfect correspondence of the sequences, but the correspondence must be sufficient to enable the RNA to direct RNAi cleavage of the target mRNA. In a particular embodiment, the 21-23 nt RNA molecules of the present invention comprise a 3' hydroxyl group.

The present invention also relates to methods of producing RNA molecules of about 21 to about 23 nucleotides with the ability to mediate RNAi cleavage. In one embodiment, the Drosophila in vitro system is used. In this embodiment, dsRNA is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA molecules of about 21 to about 23 nucleotides. In another embodiment, the Drosophila in vitro system is used to obtain RNA sequences of about 21 to about 23 nucleotides which mediate RNA interference of the mRNA of a particular gene (e.g., oncogene, viral gene). In this embodiment, double-stranded RNA that corresponds to a sequence of the gene to be targeted is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides in length. As shown herein, 21- 23 nt RNA mediates RNAi of the mRNA of the targeted gene (the gene whose mRNA is to be degraded). The method of obtaining 21-23 nt RNAs using the Drosophila in vitro system can further comprise isolating the RNA sequence from the combination.

The present invention also relates to 21-23 nt RNA produced by the methods of the present invention, as well as to 21-23 nt RNAs, produced by other methods, such as chemical synthesis or recombinant DNA techniques, that have the same or substantially the same sequences as naturally-occurring RNAs that mediate RNAi, such as those produced by the methods of the present invention. All of these are referred to as 21-23 nt RNAs that mediate RNA interference. As used herein, the term isolated RNA includes RNA obtained by any means, including processing or cleavage of dsRNA as described herein; production by chemical synthetic methods; and production by recombinant DNA techniques. The invention further relates to uses of the 21-23 nt RNAs, such as for therapeutic or prophylactic treatment and compositions comprising 21-23 nt RNAs that mediate RNAi, such as pharmaceutical compositions comprising 21-23 nt RNAs and an appropriate carrier (e.g., a buffer or water).

The present invention also relates to a method of mediating RNA interference of mRNA of a gene in a cell or organism (e.g., mammal such as a mouse or a human). In one embodiment, RNA of about 21 to about 23 nt which targets the mRNA to be degraded is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of the mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism. The cell or organism can be one in which RNAi occurs as the cell or organism is obtained or a cell or organism can be one that has been modified so that RNAi occurs (e.g., by addition of components obtained from a cell or cell extract that mediate RNAi or activation of endogenous components). As used herein, the term "cell or organism in which RNAi occurs" includes both a cell or organism in which RNAi occurs as the cell or organism is obtained, or a cell or organism that has been modified so that RNAi occurs. In another embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNAs of about 21 to about 23 nucleotides. 21 to 23 nt RNA is then

isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the gene in the cell or organism. As described for the previous embodiment, the cell or organism is one in which RNAi occurs naturally (in the cell or organism as obtained) or has been modified in such a manner that RNAi occurs. 21 to 23 nt RNAs can also be produced by other methods, such as chemical synthetic methods or recombinant DNA techniques.

The present invention also relates to biochemical components of a cell, such as a Drosophila cell, that process dsRNA to RNA of about 21 to about 23 nucleotides. In addition, biochemical components of a cell that are involved in targeting of mRNA by RNA of about 21 to about 23 nucleotides are the subject of the present invention. In both embodiments, the biochemical components can be obtained from a cell in which they occur or can be produced by other methods, such as chemical synthesis or recombinant DNA methods. As used herein, the term "isolated" includes materials (e.g., biochemical components, RNA) obtained from a source in which they occur and materials produced by methods such as chemical synthesis or recombinant nucleic acid (DNA, RNA) methods.

The present invention also relates to a method for knocking down (partially or completely) the targeted gene, thus providing an alternative to presently available methods of knocking down (or out) a gene or genes. This method of knocking down gene expression can be used therapeutically or for research (e.g., to generate models of disease states, to examine the function of a gene, to assess whether an agent acts on a gene, to validate targets for drug discovery). In those instances in which gene function is eliminated, the resulting cell or organism can also be referred to as a knockout. One embodiment of the method of producing knockdown cells and organisms comprises introducing into a cell or organism in which a gene (referred to as a targeted gene) is to be knocked down, RNA of about 21 to about 23 nt that targets the gene and maintaining the resulting cell or organism under conditions under which RNAi occurs, resulting in degradation of the mRNA of the targeted gene, thereby producing knockdown cells or organisms. Knockdown cells and organisms produced by the present method are also the subject of this invention.

The present invention also relates to a method of examining or assessing the function of a gene in a cell or organism. In one embodiment, RNA of about 21 to about 23 nt which targets mRNA of the gene for degradation is introduced into a cell or organism in which RNAi occurs. The cell or organism is referred to as a test cell or organism. The test cell or organism is maintained under conditions under which degradation of mRNA of the gene occurs. The phenotype of the test cell or organism is then observed and compared to that of an appropriate control cell or organism, such as a corresponding cell or organism that is treated in the same manner except that the targeted (specific) gene is not targeted. A 21 to 23 nt RNA that does not target the mRNA for degradation can be introduced into the control cell or organism in place of the RNA introduced into the test cell or organism, although it is not necessary to do so. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the degraded mRNA. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract that mediates RNAi, such as the soluble extract derived from Drosophila embryo described herein, under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into a cell or organism in which RNAi occurs (test cell or test organism). The test cell or test organism is maintained under conditions under which degradation of the mRNA occurs. The phenotype of the test cell or organism is then observed and compared to that of an appropriate control, such as a corresponding cell or organism that is treated in the same manner as the test cell or organism except that the targeted gene is not targeted. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the targeted gene. The information provided may be sufficient to identify (define) the function of the gene or may be used in conjunction with information obtained from other assays or analyses to do so.

Also the subject of the present invention is a method of validating whether an agent acts on a gene. In this method, RNA of from about 21 to about 23 nucleotides that targets the mRNA to be degraded is introduced into a cell or

organism in which RNAi occurs. The cell or organism (which contains the introduced RNA) is maintained under conditions under which degradation of mRNA occurs, and the agent is introduced into the cell or organism. Whether the agent has an effect on the cell or organism is determined; if the agent has no effect on the cell or organism, then the agent acts on the gene.

The present invention also relates to a method of validating whether a gene product is a target for drug discovery or development. RNA of from about 21 to about 23 nucleotides that targets the mRNA that corresponds to the gene for degradation is introduced into a cell or organism. The cell or organism is maintained under conditions in which degradation of the mRNA occurs, resulting in decreased expression of the gene. Whether decreased expression of the gene has an effect on the cell or organism is determined, wherein if decreased expression of the gene has an effect, then the gene product is a target for drug discovery or development.

The present invention also encompasses a method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNA of from about 21 to about 23 nucleotides which targets the mRNA of the protein (the mRNA that encodes the protein) for degradation. As a result, the protein is not produced or is not produced to the extent it would be in the absence of the treatment.

Also encompassed by the present invention is a gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

Also encompassed by the present invention is a method of identifying target sites within an mRNA that are particularly suitable for RNAi as well as a method of assessing the ability of 21-23 nt RNAs to mediate RNAi.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1 is a schematic representation of reporter mRNAs and dsRNAs Rr-Luc and Pp-Luc. Lengths and positions of the ssRNA, asRNA, and dsRNAs are shown as black bars relative to the Rr-Luc and Pp-Luc reporter mRNA sequences. Black rectangles indicate the two unrelated luciferase coding sequences, lines correspond to the 5' and 3' untranslated regions of the mRNAs.

Figure 2A is a graph of the ratio of luciferase activities after targeting 50 pM Pp- Luc mRNA with 10 nM ssRNA, asRNA, or dsRNA from the 505 bp segment of the Pp- Luc gene showing gene-specific interference by dsRNA in vitro. The data are the average values of seven trials ± standard deviation. Four independently prepared lysates were used. Luciferase activity was normalized to the buffer control; a ratio equal to one indicates no gene-specific interference.

Figure 2B is a graph of the ratio of luciferase activities after targeting 50 pM Rr- Luc mRNA with 10 nM ssRNA, asRNA, or dsRNA from the 501 bp segment of the Rr- Luc gene showing gene-specific interference by dsRNA in vitro. The data are the average values of six trials ± standard deviation. A Rr-Luc/Pp-Luc ratio equal to one indicates no gene-specific interference.

Figure 3A is a schematic representation of the experimental strategy used to show that incubation in the Drosophila embryo lysate potentiates dsRNA for gene-specific interference. The same dsRNAs used in Figure 2 (or buffer) was serially preincubated using two-fold dilutions in six successive reactions with Drosophila embryo lysate, then tested for its capacity to block mRNA expression. As a control, the same amount of dsRNA (10 nM) or buffer was diluted directly in buffer and incubated with Pp-Luc and Rr-Luc mRNAs and lysate.

Figure 3B is a graph of potentiation when targeting Pp-Luc mRNA. Black columns indicate the dsRNA or the buffer was serially preincubated; white columns correspond to a direct 32-fold dilution of the dsRNA. Values were normalized to those of the buffer controls.

Figure 3C is a graph of potentiation when targeting Rr-Luc mRNA. The corresponding buffer control is shown in Figure 3B.

Figure 4 is a graph showing effect of competitor dsRNA on gene-specific interference. Increasing concentrations of nanos dsRNA (508 bp) were added to

reactions containing 5 nM dsRNA (the same dsRNAs used in Figures 2A and 2B) targeting Pp-Luc mRNA (black columns, left axis) or Rr-Luc mRNA (white columns, right axis). Each reaction contained both a target mRNA (Pp-Luc for the black columns, Rr-Luc for the white) and an unrelated control mRNA (Rr-Luc for the black columns, Pp-Luc for the white). Values were normalized to the buffer control (not shown). The reactions were incubated under standard conditions (see Methods).

Figure 5A is a graph showing the effect of dsRNA on mRNA stability. Circles, Pp-Luc mRNA; squares, Rr-Luc mRNA; filled symbols, buffer incubation; open symbols, incubation with Pp-dsRNA.

Figure 5B is a graph showing the stability of Rr-Luc mRNA incubated with Rr- dsRNA or Pp-dsRNA. Filled squares, buffer; open squares, Pp-dsRNA (10 nM); open circles, Rr-dsRNA (10 nM).

Figure 5C is a graph showing the dependence on dsRNA length. The stability of the Pp-Luc mRNA was assessed after incubation in lysate in the presence of buffer or dsRNAs of different lengths. Filled squares, buffer; open circles, 49 bp dsRNA (10 nM); open inverted triangles, 149 bp dsRNA (10 nM); open triangles, 505 bp dsRNA (10 nM); open diamonds, 997 bp dsRNA (10 nM). Reactions were incubated under standard conditions (see Methods).

Figure 6 is a graph showing that RNAi Requires ATP. Creatine kinase (CK) uses creatine phosphate (CP) to regenerate ATP. Circles, +ATP, +CP, +CK; squares, -ATP, +CP, +CK; triangles, -ATP, -CP, +CK; inverted triangles, -ATP, +CP, -CK.

Figure 7A is a graph of protein synthesis, as reflected by luciferase activity produced after incubation of Rr-luc mRNA in the in vitro RNAi reaction for 1 hour, in the presence of the protein synthesis inhibitors anisomycin, cycloheximide, or chloramphenicol, relative to a reaction without any inhibitor showing that RNAi does not require mRNA translation.

Figure 7B is a graph showing translation of 7-methyl-guanosine- and adenosine- capped Pp-luc mRNAs (circles and squares, respectively) in the RNAi

reaction in the absence of dsRNA, as measured by luciferase activity produced in a one-hour incubation.

Figure 7C is a graph showing incubation in an RNAi reaction of uniformly ^{32}P- radiolabeled 7-methyl-guanosine-capped Pp-luc mRNA (circles) and adenosine-capped Pp-luc mRNA (squares), in the presence (open symbols) and absence (filled symbols) of 505 bp Pp-luc dsRNA.

Figure 8A is a graph of the of the denaturing agarose-gel analysis of Pp-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt Pp-asRNA, or 505 bp Pp-dsRNA for the times indicated showing that asRNA causes a small amount of RNAi in vitro.

Figure 8B is a graph of the of the denaturing agarose-gel analysis of Rr-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt Pp-asRNA, or 505 bp Pp-dsRNA for the times indicated showing that asRNA causes a small amount of RNAi in vitro.

Figure 9 is a schematic of the positions of the three dsRNAs, 'A,' 'B,' and 'C,' relative to the Rr-luc mRNA.

Figure10 indicates the cleavage sites mapped onto the first 267 nt of the Rr-luc mRNA (SEQ ID NO: 1). The blue bar below the sequence indicates the position of dsRNA 'C,' and blue circles indicate the position of cleavage sites caused by this dsRNA. The green bar denotes the position of dsRNA 'B,' and green circles, the cleavage sites. The magenta bar indicates the position of dsRNA 'A,' and magenta circles, the cleavages. An exceptional cleavage within a run of 7 uracils is marked with a red arrowhead.

Figure 11 is a proposed model for RNAi. RNAi is envisioned to begin with cleavage of the dsRNA to 21-23 nt products by a dsRNA-specific nuclease, perhaps in a multiprotein complex. These short dsRNAs might then be dissociated by an ATP- dependent helicase, possibly a component of the initial complex, to 21-23 nt asRNAs that could then target the mRNA for cleavage. The short asRNAs are imagined to remain associated with the RNAi-specific proteins (circles) that were originally bound by the full-length dsRNA, thus explaining the inefficiency of

asRNA to trigger RNAi in vivo and in vitro. Finally, a nuclease (triangles) would cleave the mRNA.

Figure 12 is a bar graph showing sequence-specific gene silencing by 21-23 nt fragments. Ratio of luciferase activity after targeting of Pp-Luc and Rr-Luc mRNA by 5 nM Pp-Luc or Rr-Luc dsRNA (500 bp) or 21-23 nt fragments isolated from a previous incubation of the respective dsRNA in Drosophila lysate. The amount of isolated 21-23 mers present in the incubation reaction correspond to approximately the same amount of 21-23 mers generated during an incubation reaction with 5 nM 500 bp dsRNA. The data are average values of 3 trials and the standard deviation is given by error bars. Luciferase activity was normalized to the buffer control.

Figure 13A illustrates the purification of RNA fragments on a Superdex HR 200 10/30 gel filtration column (Pharmacia) using the method described in Example 4. dsRNA was 32P-labeled, and the radioactivity recovered in each column fraction is graphed. The fractions were also analyzed by denaturing gel electrophoresis (inset).

Figure 13B demonstrates the ability of the Rr-luciferase RNA, after incubation in the Drosophila lysate and fractionation as in Fig. 13A, to mediate sequence-specific interference with the expression of a Rr-luciferase target mRNA. One microliter of each resuspended fraction was tested in a 10 microliter in vitro RNAi reaction (see Example 1). This procedure yields a concentration of RNA in the standard in vitro RNAi reaction that is approximately equal to the concentration of that RNA species in the original reaction prior to loading on the column. Relative luminescence per second has been normalized to the average value of the two buffer controls.

Figure 13C is the specificity control for Fig 13B. It demonstrates that the fractionated RNA of Fig 13B does not efficiently mediate sequence-specific interference with the expression of a Pp-luciferase mRNA. Assays are as in Fig 13B.

Figures 14A and 14B are schematic representations of reporter constructs and siRNA duplexes. Figure 14A illustrates the firefly (*Pp*-luc) and sea pansy (*Rr*-

luc) luciferase reporter gene regions from plasmids pGL2-Control, pGL3-Control, and pRL-TK (Promega). SV40 regulatory elements, the HSV thymidine kinase promoter, and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase expression from pGL2 is approximately 10-fold lower than from pGL3 in transfected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes. Figure 14B shows the sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2 (SEQ ID Nos: 10 and 11), GL3 (SEQ ID Nos: 12 and 13), and RL (SEQ ID Nos: 14 and 15) luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only 3 single nucleotide substitutions (boxed in gray). As unspecific control, a duplex with the inverted GL2 sequence, invGL2 (SEQ ID Nos: 16 and 17), was synthesized. The 2 nt 3' overhang of 2'-deoxythymidine is indicated as TT; uGL2 (SEQ ID Nos: 18 and 19) is similar to GL2 siRNA but contains ribo-uridine 3' overhangs.

Figures 15A-15J are graphs showing RNA interference by siRNA duplexes. Ratios of target to control luciferase were normalized to a buffer control (bu, black bars); gray bars indicate ratios of *Photinus pyralis* (*Pp*-luc) GL2 or GL3 luciferase to *Renilla reniformis* (*Rr*-luc) RL luciferase (left axis), white bars indicate RL to GL2 or GL3 ratios (right axis). Figures 15A, 15C, 15E, 15G, and 15I show results of experiments performed with the combination of pGL2-Control and pRL-TK reporter plasmids, Figures 15B, 15D, 15F, 15H, and 15J with pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of *Pp*-luc/*Rr*-luc for the buffer control (bu) varied between 0.5 and 10 for pGL2/pRL, and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and between the various cell lines tested. The plotted data were averaged from three independent experiments ± S.D.

Figures 16A-16F are graphs showing the effects of 21 nt siRNAs, 50 bp, and 500 bp dsRNAs on luciferase expression in HeLa cells. The exact length of the long dsRNAs is indicated below the bars. Figures 16A, 16C, and 16E describe experiments performed with pGL2-Control and pRL-TK reporter plasmids, Figures 16B, 16D, and 16F with pGL3-Control and pRL-TK reporter plasmids. The data

were averaged from two independent experiments ± S.D. Figures 16A, 16B, Absolute *Pp*-luc expression, plotted in arbitrary luminescence units. Figure 16C, 16D, Rr-luc expression, plotted in arbitrary luminescence units. Figures 16E, 16F, Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (bu, black bars); the luminescence ratios for 50 or 500 bp dsRNAs were normalized to the respective ratios observed for 50 and 500 bp dsRNA from humanized GFP (hG, black bars). It should be noted, that the overall differences in sequence between the 49 and 484 bp dsRNAs targeting GL2 and GL3 are not sufficient to confer specificity between GL2 and GL3 targets (43 nt uninterrupted identity in 49 bp segment, 239 nt longest uninterrupted identity in 484 bp segment) (Parrish, S., *et al., Mol. Cell, 6:*1077-1087 (2000)).

DETAILED DESCRIPTION OF THE INVENTION

Double-stranded (dsRNA) directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). The process is known to occur in a wide variety of organisms, including embryos of mammals and other vertebrates. Using the Drosophila in vitro system described herein, it has been demonstrated that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length, and furthermore, that when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of longer dsRNA. Thus, these 21-23 nt fragments are sequence-specific mediators of RNA degradation. A molecular signal, which may be the specific length of the fragments, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or recombinantly produced or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells. Use of long dsRNAs in mammalian cells to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. Specific targeting of a particular gene function, which is possible with

21-23 nt fragments of the present invention, is useful in functional genomic and therapeutic applications.

In particular, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that mediate RNAi. In one embodiment, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that direct cleavage of specific mRNA to which they correspond. The 21-23 nt RNA molecules of the present invention can also comprise a 3' hydroxyl group. The 21-23 nt RNA molecules can be single-stranded or double stranded (as two 21-23 nt RNAs); such molecules can be blunt ended or comprise overhanging ends (*e.g.*, 5', 3'). In specific embodiments, the RNA molecule is double stranded and either blunt ended or comprises overhanging ends (as two 21-23 nt RNAs).

In one embodiment, at least one strand of the RNA molecule has a 3' overhang from about 1 to about 6 nucleotides (*e.g.*, pyrimidine nucleotides, purine nucleotides) in length. In other embodiments, the 3' overhang is from about 1 to about 5 nucleotides, from about 1 to about 3 nucleotides and from about 2 to about 4 nucleotides in length. In one embodiment the RNA molecule is double stranded, one strand has a 3' overhang and the other strand can be blunt-ended or have an overhang. In the embodiment in which the RNA molecule is double stranded and both strands comprise an overhang, the length of the overhangs may be the same or different for each strand. In a particular embodiment, the RNA of the present invention comprises 21 nucleotide strands which are paired and which have overhangs of from about 1 to about 3, particularly about 2, nucleotides on both 3' ends of the RNA. In order to further enhance the stability of the RNA of the present invention, the 3' overhangs can be stabilized against degradation. In one embodiment, the RNA is stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, *e.g.*, substitution of uridine 2 nucleotide 3' overhangs by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNAi. The absence of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium.

The 21-23 nt RNA molecules of the present invention can be obtained using a number of techniques known to those of skill in the art. For example, the RNA can be chemically synthesized or recombinantly produced using methods known in the art. The 21-23 nt RNAs can also be obtained using the Drosophila in vitro system described herein. Use of the Drosophila in vitro system entails combining dsRNA with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA of about 21 to about 23 nucleotides. The Drosophila in vitro system can also be used to obtain RNA of about 21 to about 23 nucleotides in length which mediates RNA interference of the mRNA of a particular gene (e.g., oncogene, viral gene). In this embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double- stranded RNA is processed to the RNA of about 21 to about 23 nucleotides. As shown herein, 21-23 nt RNA mediates RNAi of the mRNA to be degraded. The present invention also relates to the 21-23 nt RNA molecules produced by the methods described herein.

In one embodiment, the methods described herein are used to identify or obtain 21-23 nt RNA molecules that are useful as sequence-specific mediators of RNA degradation and, thus, for inhibiting mRNAs, such as human mRNAs, that encode products associated with or causative of a disease or an undesirable condition. For example, production of an oncoprotein or viral protein can be inhibited in humans in order to prevent the disease or condition from occurring, limit the extent to which it occurs or reverse it. If the sequence of the gene to be targeted in humans is known, 21-23 nt RNAs can be produced and tested for their ability to mediate RNAi in a cell, such as a human or other primate cell. Those 21-23 nt human RNA molecules shown to mediate RNAi can be tested, if desired, in an appropriate animal model to further assess their in vivo effectiveness. Additional copies of 21-23 nt RNAs shown to mediate RNAi can be produced by the methods described herein.

The method of obtaining the 21-23 nt RNA sequence using the Drosophila in vitro system can further comprise isolating the RNA sequence from the combination. The 21-23 nt RNA molecules can be isolated using a number of techniques known to those of skill in the art. For example, gel electrophoresis can be used to separate 21-23 nt RNAs from the combination, gel slices comprising the RNA sequences removed and RNAs eluted from the gel slices. Alternatively, non-denaturing methods, such as non-denaturing column chromatography, can be used to isolate the RNA produced. In addition, chromatography (e.g., size exclusion chromatography), glycerol gradient centrifugation, affinity purification with antibody can be used to isolate 21-23 nt RNAs. The RNA-protein complex isolated from the Drosophila in vitro system can also be used directly in the methods described herein (e.g., method of mediating RNAi of mRNA of a gene). Soluble extracts derived from Drosophila embryo that mediate or RNAi are encompassed by the invention. The soluble Drosophila extract can be obtained in a variety of ways. For example, the soluble extract can be obtained from syncytial blastoderm Drosophila embryos as described in Examples 1, 2, and 3. Soluble extracts can be derived from other cells in which RNAi occurs. Alternatively, soluble extracts can be obtained from a cell that does not carry out RNAi. In this instance, the factors needed to mediate RNAi can be introduced into such a cell and the soluble extract is then obtained. The components of the extract can also be chemically synthesized and/or combined using methods known in the art.

Any dsRNA can be used in the methods of the present invention, provided that it has sufficient homology to the targeted gene to mediate RNAi. The sequence of the dsRNA for use in the methods of the present invention need not be known. Alternatively, the dsRNA for use in the present invention can correspond to a known sequence, such as that of an entire gene (one or more) or portion thereof. There is no upper limit on the length of the dsRNA that can be used. For example, the dsRNA can range from about 21 base pairs (bp) of the gene to the full length of the gene or more. In one embodiment, the dsRNA used in the methods of the present invention is about 1000 bp in length. In another embodiment, the dsRNA is about 500 bp in length. In yet another embodiment, the dsRNA is about 22 bp in length.

The 21 to 23 nt RNAs described herein can be used in a variety of ways. For example, the 21 to 23 nt RNA molecules can be used to mediate RNA interference of mRNA of a gene in a cell or organism. In a specific embodiment, the 21 to 23 nt RNA is introduced into human cells or a human in order to mediate RNA interference in the cells or in cells in the individual, such as to prevent or treat a disease or undesirable condition. In this method, a gene (or genes) that cause or contribute to the disease or undesirable condition is targeted and the corresponding mRNA (the transcriptional product of the targeted gene) is degraded by RNAi. In this embodiment, an RNA of about 21 to about 23 nucleotides that targets the corresponding mRNA (the mRNA of the targeted gene) for degradation is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of the corresponding mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism. In a particular embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides. The 21 to 23 nt RNA is then isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the gene in the cell or organism. In the event that the 21-23nt RNA is introduced into a cell in which RNAi, does not normally occur, the factors needed to mediate RNAi are introduced into such a cell or the expression of the needed factors is induced in such a cell. Alternatively, 21 to 23 nt RNA produced by other methods (e.g., chemical synthesis, recombinant DNA production) to have a composition the same as or sufficiently similar to a 21 to 23 nt RNA known to mediate RNAi can be similarly used to mediate RNAi. Such 21 to 23 nt RNAs can be altered by addition, deletion, substitution or modification of one or more nucleotides and/or can comprise non-nucleotide materials. A further embodiment of this invention is an ex vivo method of treating cells from an individual to degrade a gene(s) that causes or is associated with a disease or

undesirable condition, such as leukemia or AIDS. In this embodiment, cells to be treated are obtained from the individual using known methods (e.g., phlebotomy or collection of bone marrow) and 21-23 nt RNAs that mediate degradation of the corresponding mRNA(s) are introduced into the cells, which are then re-introduced into the individual. If necessary, biochemical components needed for RNAi to occur can also be introduced into the cells.

The mRNA of any gene can be targeted for degradation using the methods of mediating interference of mRNA described herein. For example, any cellular or viral mRNA, can be targeted, and, as a result, the encoded protein (e.g., an oncoprotein, a viral protein), expression will be diminished. In addition, the mRNA of any protein associated with/causative of a disease or undesirable condition can be targeted for degradation using the methods described herein.

The present invention also relates to a method of examining the function of a gene in a cell or organism. In one embodiment, an RNA sequence of about 21 to about 23 nucleotides that targets mRNA of the gene for degradation is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of mRNA of the gene occurs. The phenotype of the cell or organism is then observed and compared to an appropriate control, thereby providing information about the function of the gene. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract derived from Drosophila embryo under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of the mRNA of the gene occurs. The phenotype of the cell or organism is then observed and compared to an appropriate control, thereby identifying the function of the gene.

A further aspect of this invention is a method of assessing the ability of 21-23 nt RNAs to mediate RNAi and, particularly, determining which 21-23 nt RNA(s) most efficiently mediate RNAi. In one embodiment of the method, dsRNA corresponding to a sequence of an mRNA to be degraded is combined with

detectably labeled (e.g., end-labeled, such as radiolabeled) mRNA and the soluble extract of this invention, thereby producing a combination. The combination is maintained under conditions under which the double-stranded RNA is processed and the mRNA is degraded. The sites of the most effective cleavage are mapped by comparing the migration of the labeled mRNA cleavage products to markers of known length. 21 mers spanning these sites are then designed and tested for their efficiency in mediating RNAi.

Alternatively, the extract of the present invention can be used to determine whether there is a particular segment or particular segments of the mRNA corresponding to a gene which are more efficiently targeted by RNAi than other regions and, thus, can be especially useful target sites. In one embodiment, dsRNA corresponding to a sequence of a gene to be degraded, labeled mRNA of the gene is combined with a soluble extract that mediates RNAi, thereby producing a combination. The resulting combination is maintained under conditions under which the dsRNA is degraded and the sites on the mRNA that are most efficiently cleaved are identified, using known methods, such as comparison to known size standards on a sequencing gel.

OVERVIEW OF EXAMPLES

Biochemical analysis of RNAi has become possible with the development of the in vitro Drosophila embryo lysate that recapitulates dsRNA-dependent silencing of gene expression described in Example 1 (Tuschl et al., Genes Dev., 13:3191-7 (1999)). In the in vitro system, dsRNA, but not sense or asRNA, targets a corresponding mRNA for degradation, yet does not affect the stability of an unrelated control mRNA. Furthermore, pre-incubation of the dsRNA in the lysate potentiates its activity for target mRNA degradation, suggesting that the dsRNA must be converted to an active form by binding proteins in the extract or by covalent modification (Tuschl et al., Genes Dev., 13:3191-7 (1999)).

The development of a cell-free system from syncytial blastoderm Drosophila embryos that recapitulates many of the features of RNAi is described herein. The interference observed in this reaction is sequence-specific, is promoted by dsRNA,

but not by single-stranded RNA, functions by specific mRNA degradation, requires a minimum length of dsRNA and is most efficient with long dsRNA. Furthermore, preincubation of dsRNA potentiates its activity. These results demonstrate that RNAi is mediated by sequence specific processes in soluble reactions.

As described in Example 2, the in vitro system was used to analyze the requirements of RNAi and to determine the fate of the dsRNA and the mRNA. RNAi in vitro requires ATP, but does not require either mRNA translation or recognition of the 7-methyl-guanosine cap of the targeted mRNA. The dsRNA, but not single-stranded RNA, is processed in vitro to a population of 21-23 nt species. Deamination of adenosines within the dsRNA does not appear to be required for formation of the 21-23 nt RNAs. As described herein, the mRNA is cleaved only in the region corresponding to the sequence of the dsRNA and that the mRNA is cleaved at 21-23 nt intervals, strongly indicating that the 21-23 nt fragments from the dsRNA are targeting the cleavage of the mRNA. Furthermore, as described in Examples 3 and 4, when the 21-23 nt fragments are purified and added back to the soluble extract, they mediate RNA.

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

Example 1 Targeted mRNA degradation by double-stranded RNA in vitro

Materials and Methods

RNAs

Rr-Luc mRNA consisted of the 926 nt Rr luciferase coding sequence flanked by 25 nt of 5' untranslated sequence from the pSP64 plasmid polylinker and 25 nt of 3' untranslated sequence consisting of 19 nt of pSP64 plasmid polylinker sequence followed by a 6 nt Sac I site. Pp-Luc mRNA contained the 1653 nt Pp luciferase coding sequence with a Kpn I site introduced immediately before the Pp luciferase stop codon. The Pp coding sequence was flanked by 5' untranslated sequences consisting of 21 nt of pSP64 plasmid polylinker followed by the 512 nt of the 5' untranslated region (UTR) from the Drosophila hunchback mRNA and 3' untranslated sequences consisting of the 562 nt hunchback 3' UTR followed by a 6

nt Sac I site. The hunchback 3' UTR sequences used contained six G-to-U mutations that disrupt function of the Nanos Response Elements in vivo and in vitro. Both reporter mRNAs terminated in a 25 nt poly(A) tail encoded in the transcribed plasmid. For both Rr-Luc and Pp -Luc mRNAs, the transcripts were generated by run-off transcription from plasmid templates cleaved at an Nsi I site that immediately followed the 25 nt encoded poly(A) tail. To ensure that the transcripts ended with a poly(A) tail, the Nsi I-cleaved transcription templates were resected with T4 DNA Polymerase in the presence of dNTPs. The SP6 mMessage mMachine kit (Ambion) was used for in vitro transcription. Using this kit, about 80% of the resulting transcripts are 7-methyl guanosine capped. ^{32}P-radiolabeling was accomplished by including α-^{32}P-UTP in the transcription reaction.

For Pp -Luc, ss, as, and dsRNA corresponded to positions 93 to 597 relative to the start of translation, yielding a 505 bp dsRNA. For Rr -Luc, ss, as, and dsRNA corresponded to positions 118 to 618 relative to the start of translation, yielding a 501 bp dsRNA. The Drosophila nanos competitor dsRNA corresponded to positions 122 to 629 relative to the start of translation, yielding a 508 bp dsRNA. ssRNA, asRNA, and dsRNA (diagrammed in Figure 1) were transcribed in vitro with T7 RNA polymerase from templates generated by the polymerase chain reaction. After gel purification of the T7 RNA transcripts, residual DNA template was removed by treatment with RQ1 DNase (Promega). The RNA was then extracted with phenol and chloroform, and then precipitated and dissolved in water.

RNA annealing and native gel electrophoresis.

ssRNA and asRNA (0.5 μM) in 10 mM Tris-HCl (pH 7.5) with 20 mM NaCl were heated to 95 ° C for 1 min then cooled and annealed at room temperature for 12 to 16 h. The RNAs were precipitated and resuspended in lysis buffer (below). To monitor annealing, RNAs were electrophoresed in a 2% agarose gel in TBE buffer and stained with ethidium bromide (Sambrook et al., Molecular Cloning. Cold Spring Harbor Laboratory Press, Plainview, NY. (1989)).

Lysate preparation

Zero- to two-hour old embryos from Oregon R flies were collected on yeasted molasses agar at 25°C. Embryos were dechorionated for 4 to 5 min in 50% (v/v) bleach, washed with water, blotted dry, and transferred to a chilled Potter-Elvehjem tissue grinder (Kontes). Embryos were lysed at 4°C in one ml of lysis buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) containing 5 mM dithiothreitol (DTT) and 1 mg/ml Pefabloc SC (Boehringer-Mannheim) per gram of damp embryos. The lysate was centrifuged for 25 min at 14,500 x g at 4°C, and the supernatant flash frozen in aliquots in liquid nitrogen and stored at -80°C.

Reaction conditions

Lysate preparation and reaction conditions were derived from those described by Hussain and Leibowitz (Hussain and Leibowitz, Gene 46:13-23 (1986)). Reactions contained 50% (v/v) lysate, mRNAs (10 to 50 pM final concentration), and 10% (v/v) lysis buffer containing the ssRNA, asRNA, or dsRNA (10 nM final concentration). Each reaction also contained 10 mM creatine phosphate, 10 μg/ml creatine phosphokinase, 100 μM GTP, 100 μM UTP, 100 μM CTP, 500 μM ATP, 5 μM DTT, 0.1 U/mL RNasin (Promega), and 100 μM of each amino acid. The final concentration of potassium acetate was adjusted to 100 mM. For standard conditions, the reactions were assembled on ice and then pre-incubated at 25° C for 10 min before adding mRNA. After adding mRNAs, the incubation was continued for an additional 60 min. The 10 min preincubation step was omitted for the experiments in Figures 3A-3C and 5A-5C. Reactions were quenched with four volumes of 1.25x Passive Lysis Buffer (Promega). Pp and Rr luciferase activity was detected in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory) using the Dual-Luciferase Reporter Assay System (Promega).

RNA stability

Reactions with ^{32}P-radiolabeled mRNA were quenched by the addition of 40 volumes of 2x PK buffer (200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 300 mM NaCl,

2% w/v sodium dodecyl sulfate). Proteinase K (E.M. Merck; dissolved in water) was added to a final concentration of 465 µg/ml. The reactions were then incubated for 15 min at 65° C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with an equal volume of isopropanol. Reactions were analyzed by electrophoresis in a formaldehyde/agarose (0.8% w/v) gel (Sambrook et al., Molecular Cloning. Cold Spring Harbor Laboratory Press, Plainview, NY. (1989)). Radioactivity was detected by exposing the agarose gel [dried under vacuum onto Nytran Plus membrane (Amersham)] to an image plate (Fujix) and quantified using a Fujix Bas 2000 and Image Gauge 3.0 (Fujix) software.

Commercial lysates

Untreated rabbit reticulocyte lysate (Ambion) and wheat germ extract (Ambion) reactions were assembled according to the manufacturer's directions. dsRNA was incubated in the lysate at 27°C (wheat germ) or 30°C (reticulocyte lysate) for 10 min prior to the addition of mRNAs.

Results and Discussion

To evaluate if dsRNA could specifically block gene expression in vitro, reporter mRNAs derived from two different luciferase genes that are unrelated both in sequence and in luciferin substrate specificity were used: Renilla reniformis (sea pansy) luciferase (Rr-Luc) and Photuris pennsylvanica (firefly) luciferase (Pp-Luc). dsRNA generated from one gene was used to target that luciferase mRNA whereas the other luciferase mRNA was an internal control co-translated in the same reaction. dsRNAs of approximately 500 bp were prepared by transcription of polymerase-chain reaction products from the Rr-Luc and Pp-Luc genes. Each dsRNA began ~100 bp downstream of the start of translation (Figure 1). Sense (ss) and anti-sense (as) RNA were transcribed in vitro and annealed to each other to produce the dsRNA. Native gel electrophoresis of the individual Rr 501 and Pp 505 nt as RNA and ssRNA used to form the Rr and Pp dsRNAs was preformed. The ssRNA, asRNA, and dsRNAs were each tested for their ability to block specifically

expression of their cognate mRNA but not the expression of the unrelated internal control mRNA.

The ssRNA, asRNA, or dsRNA was incubated for 10 min in a reaction containing Drosophila embryo lysate, then both Pp-Luc and Rr-Luc mRNAs were added and the incubation continued for an additional 60 min. The Drosophila embryo lysate efficiently translates exogenously transcribed mRNA under the conditions used. The amounts of Pp-Luc and Rr-Luc enzyme activities were measured and were used to calculate ratios of either Pp-Luc/Rr-Luc (Figure 2A) or Rr-Luc/Pp-Luc (Figure 2B). To facilitate comparison of different experiments, the ratios from each experiment were normalized to the ratio observed for a control in which buffer was added to the reaction in place of ssRNA, asRNA, or dsRNA.

Figure 2A shows that a 10 nM concentration of the 505 bp dsRNA identical to a portion of the sequence of the Pp-Luc gene specifically inhibited expression of the Pp- Luc mRNA but did not affect expression of the Rr-Luc internal control. Neither ssRNA nor asRNA affected expression of Pp-Luc or the Rr-Luc internal control. Thus, Pp-Luc expression was specifically inhibited by its cognate dsRNA. Conversely, a 10 nM concentration of the 501 bp dsRNA directed against the Rr-Luc mRNA specifically inhibited Rr-Luc expression but not that of the Pp-Luc internal control (Figure 2B). Again, comparable levels of ssRNA or asRNA had little or no effect on expression of either reporter mRNA. On average, dsRNA reduced specific luciferase expression by 70% in these experiments, in which luciferase activity was measured after 1 h incubation. In other experiments in which the translational capacity of the reaction was replenished by the addition of fresh lysate and reaction components, a further reduction in targeted luciferase activity relative to the internal control was observed.

The ability of dsRNA but not asRNA to inhibit gene expression in these lysates is not merely a consequence of the greater stability of the dsRNA (half-life about 2 h) relative to the single-stranded RNAs (half-life ~ 10 min). ssRNA and asRNA transcribed with a 7-methyl guanosine cap were as stable in the lysate as uncapped dsRNA, but do not inhibit gene expression. In contrast, dsRNA formed

from the capped ssRNA and asRNA specifically blocks expression of the targeted mRNA.

Effective RNAi in Drosophila requires the injection of about 0.2 fmol of dsRNA into a syncytial blastoderm embryo (Kennerdell and Carthew, Cell 95:1017-1026 (1998); Carthew, www1.pitt.edu/~carthew/manual/RNAi_Protocol.html (1999)). Since the average volume of a Drosophila embryo is approximately 7.3 nl, this corresponds to an intracellular concentration of about 25 nM (Mazur et al., Cryobiology 25:543-544 (1988)). Gene expression in the Drosophila lysate was inhibited by a comparable concentration of dsRNA (10 nM), but lowering the dsRNA concentration ten-fold decreased the amount of specific interference. Ten nanomolar dsRNA corresponds to a 200-fold excess of dsRNA over target mRNA added to the lysate. To test if this excess of dsRNA might reflect a time- and/or concentration-dependent step in which the input dsRNA was converted to a form active for gene-specific interference, the effect of preincubation of the dsRNA on its ability to inhibit expression of its cognate mRNA was examined. Because the translational capacity of the lysates is significantly reduced after 30 min of incubation at 25°C (unpublished observations), it was desired to ensure that all factors necessary for RNAi remained active throughout the pre-incubation period. Therefore, every 30 min, a reaction containing dsRNA and lysate was mixed with a fresh reaction containing unincubated lysate (Figure 3A). After six successive serial transfers spanning 3 hours of preincubation, the dsRNA, now diluted 64-fold relative to its original concentration, was incubated with lysate and 50 pM of target mRNA for 60 min. Finally, the Pp-Luc and Rr-Luc enzyme levels were measured. For comparison, the input amount of dsRNA (10 nM) was diluted 32-fold in buffer, and its capacity to generate gene-specific dsRNA interference in the absence of any preincubation step was assessed.

The preincubation of the dsRNA in lysate significantly potentiated its capacity to inhibit specific gene expression. Whereas the dsRNA diluted 32-fold showed no effect, the preincubated dsRNA was, within experimental error, as potent as undiluted dsRNA, despite having undergone a 64-fold dilution. Potentiation of

the dsRNA by preincubation was observed for dsRNAs targeting both the Pp-Luc mRNA (Figure 3B) and the Rr-Luc mRNA (Figure 3C). Taking into account the 64-fold dilution, the activation conferred by preincubation allowed a 156 pM concentration of dsRNA to inhibit 50 pM target mRNA. Further, dilution of the "activated" dsRNA may be effective but has not been tested. We note that although both dsRNAs tested were activated by the preincubation procedure, each fully retained its specificity to interfere with expression only of the mRNA to which it is homologous. Further study of the reactions may provide a route to identifying the mechanism of dsRNA potentiation.

One possible explanation for the observation that preincubation of the dsRNA enhances its capacity to inhibit gene expression in these lysates is that specific factors either modify and/or associate with the dsRNA. Accordingly, the addition of increasing amounts of dsRNA to the reaction might titrate such factors and decrease the amount of gene-specific interference caused by a second dsRNA of unrelated sequence. For both Pp-Luc mRNA and Rr-Luc mRNA, addition of increasing concentrations of the unrelated Drosophila nanos dsRNA to the reaction decreased the amount of gene- specific interference caused by dsRNA targeting the reporter mRNA (Figure 4). None of the tested concentrations of nanos dsRNA affected the levels of translation of the untargeted mRNA, demonstrating that the nanos dsRNA specifically titrated factors involved in gene-specific interference and not components of the translational machinery. The limiting factor(s) was titrated by addition of approximately 1000 nM dsRNA, a 200-fold excess over the 5 nM of dsRNA used to produce specific interference.

Interference in vitro might reflect either a specific inhibition of mRNA translation or the targeted destruction of the specific mRNA. To distinguish these two possibilities, the fates of the Pp-Luc and Rr-Luc mRNAs were examined directly using ^{32}P-radiolabeled substrates. Stability of 10 nM Pp-Luc mRNA or Rr-Luc mRNA incubated in lysate with either buffer or 505 bp Pp-dsRNA (10 nM). Samples were deproteinized after the indicated times and the ^{32}P-radiolabeled mRNAs were then resolved by denaturing gel electrophoresis. In the absence of dsRNA, both the Pp-Luc and Rr-Luc mRNAs were stable in the lysates, with ~ 75%

of the input mRNA remaining after 3 h of incubation. (About 25% of the input mRNA is rapidly degraded in the reaction and likely represents uncapped mRNA generated by the in vitro transcription process.) In the presence of dsRNA (10 nM, 505 bp) targeting the Pp-Luc mRNA, less than 15% of the Pp-Luc mRNA remained after 3 h (Figure 5A). As expected, the Rr-Luc mRNA remained stable in the presence of the dsRNA targeting Pp-Luc mRNA. Conversely, dsRNA (10 nM, 501 bp) targeting the Rr-Luc mRNA caused the destruction of the Rr-Luc mRNA but had no effect on the stability of Pp-Luc mRNA (Figure 5B). Thus, the dsRNA specifically caused accelerated decay of the mRNA to which it is homologous with no effect on the stability of the unrelated control mRNA. This finding indicates that in vivo, at least in Drosophila, the effect of dsRNA is to directly destabilize the target mRNA, not to change the subcellular localization of the mRNA, for example, by causing it to be specifically retained in the nucleus, resulting in non-specific degradation.

These results are consistent with the observation that RNAi leads to reduced cytoplasmic mRNA levels in vivo, as measured by in situ hybridization (Montgomery et al., Proc. Natl. Acad. Sci. USA 95:15502-15507 (1998)) and Northern blotting (Ngo et al., Proc. Natl. Acad. Sci. USA 95:14687-14692 (1998)). Northern blot analyses in trypanosomes and hydra suggest that dsRNA typically decreases mRNA levels by less than 90% (Ngo et al., Proc. Natl. Acad. Sci. USA 95:14687-14692 (1998); Lohmann et al., Dev. Biol. 214:211-214 (1999)). The data presented here show that in vitro mRNA levels are reduced 65 to 85% after three hours incubation, an effect comparable with observations in vivo. They also agree with the finding that RNAi in C. elegans is post- transcriptional (Montgomery et al., Proc. Natl. Acad. Sci. USA 95:15502-15507 (1998)). The simplest explanation for the specific effects on protein synthesis is that it reflects the accelerated rate of RNA decay. However, the results do not exclude independent but specific effects on translation as well as stability.

In vivo, RNAi appears to require a minimum length of dsRNA (Ngo et al., Proc. Natl. Acad. Sci., USA, 95:14687-14692 (1998)). The ability of RNA duplexes of lengths 49 bp, 149 bp, 505 bp, and 997 bp (diagrammed in Figure 1) to target the

degradation of the Pp-Luc mRNA in vitro was assessed. In good agreement with in vivo observations, the 49 bp dsRNA was ineffective in vitro, while the 149 bp dsRNA enhanced mRNA decay only slightly, and both the 505 and 997 bp dsRNAs caused robust mRNA degradation (Figure 5C). 50bp dsRNA targeting other portions of the mRNA cause detectable mRNA degradation, though not as robust as that seen for 500bp dsRNA. Thus, although some short dsRNA do not mediate RNAi, others of approximately the same length, but different composition, will be able to do so.

Whether the gene-specific interference observed in Drosophila lysates was a general property of cell-free translation systems was examined. The effects of dsRNAs on expression of Pp-Luc and Rr-Luc mRNA were examined in commercially available wheat germ extracts and rabbit reticulocyte lysates. There was no effect of addition of 10 nM of either ssRNA, asRNA, or dsRNA on the expression of either mRNA reporter in wheat germ extracts. In contrast, the addition of 10 nM of dsRNA to the rabbit reticulocyte lysate caused a profound and rapid, non-specific decrease in mRNA stability. For example, addition of Rr-Luc dsRNA caused degradation of both Rr-Luc and Pp-Luc mRNAs within 15 min. The same non-specific effect was observed upon addition of Pp-Luc dsRNA. The non-specific destruction of mRNA induced by the addition of dsRNA to the rabbit reticulocyte lysate presumably reflects the previously observed activation of RNase L by dsRNA (Clemens and Williams, Cell 13:565-572 (1978); Williams et al., Nucleic Acids Res. 6:1335-1350 (1979); Zhou et al., Cell 72:753-765 (1993); Matthews, Interactions between Viruses and the Cellular Machinery for Protein Synthesis. In Translational Control (eds. J. Hershey, M. Mathews and N. Sonenberg), pp. 505-548. Cold Spring Harbor Laboratory Press, Plainview, NY. (1996)). Mouse cell lines lacking dsRNA-induced anti-viral pathways have recently been described (Zhou et al., Virology 258:435-440 (1999)) and may be useful in the search for mammalian RNAi. Although RNAi is known to exist in some mammalian cells (Wianny and Zernicka-Goetz Nat. Cell Biol. 2: 70-75 (2000)), in many mammalian cell types its presence is likely obscured by the rapid induction by dsRNA of non-specific anti-viral responses.

dsRNA-targeted destruction of specific mRNA is characteristic of RNAi, which has been observed in vivo in many organisms, including Drosophila. The system described above recapitulates in a reaction in vitro many aspects of RNAi. The targeted mRNA is specifically degraded whereas unrelated control mRNAs present in the same solution are not affected. The process is most efficient with dsRNAs greater than 150 bp in length. The dsRNA-specific degradation reaction in vitro is probably general to many, if not all, mRNAs since it was observed using two unrelated genes.

The magnitude of the effects on mRNA stability in vitro described herein are comparable with those reported in vivo (Ngo et al., Proc. Natl. Acad. Sci., USA, 95:14687-14692 (1998); Lohmann et al., Dev. Biol., 214:211-214 (1999). However, the reaction in vitro requires an excess of dsRNA relative to mRNA. In contrast, a few molecules of dsRNA per cell can inhibit gene expression in vivo (Fire et al., Nature, 391: 806-811 (1998); Kennerdell and Carthew, Cell, 95:1017-1026 (1998)). The difference between the stoichiometry of dsRNA to target mRNA in vivo and in vitro should not be surprising in that most in vitro reactions are less efficient than their corresponding in vivo processes. Interestringly, incubation of the dsRNA in the lysate greatly potentiated its activity for RNAi, indicating that it is either modified or becomes associated with other factors or both. Perhaps a small number of molecules is effective in inhibiting the targeted mRNA in vivo because the injected dsRNA has been activated by a process similar to that reported here for RNAi in Drosophila lysates.

Example 2 Double-Stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals

Methods and Material

In vitro RNAi

In vitro RNAi reactions and lysate preparation were as described in Example 1 (Tuschl et al., Genes Dev., 13:3191-7 (1999)) except that the reaction contained 0.03 g/ml creatine kinase, 25 μM creatine phosphate (Fluka), and 1 mM ATP.

Creatine phosphate was freshly dissolved at 500 mM in water for each experiment. GTP was omitted from the reactions, except in Figures 2 and 3.

RNA Synthesis.

Pp-luc and Rr-luc mRNAs and Pp- and Rr-dsRNAs (including dsRNA 'B' in Figure 6) were synthesized by in vitro transcription as described previously (Tuschl et al., Genes Dev., 13:3191-7 (1999)). To generate transcription templates for dsRNA 'C,' the 5′ sense RNA primer was gcgtaatacgactcactataGAACAAAGGAAACGGATGAT (SEQ ID NO: 2) and the 3′ sense RNA primer was GAAGAAGTTATTCTCCAAAA (SEQ ID NO: 3); the 5′ asRNA primer was gcgtaatacgactcactataGAAGAAGTTATTCTCCAAAA (SEQ ID NO: 4)and the 3′ asRNA primer was GAACAAAGGAAACGGATGAT (SEQ ID NO: 5). For dsRNA 'A' the 5′ sense RNA primer was gcgtaatacgactcactataGTAGCGCGGTGTATTATACC (SEQ ID NO: 6)and the 3′ sense RNA primer was GTACAACGTCAGGTTTACCA (SEQ ID NO: 7); the 5′ asRNA primer was gcgtaatacgactcactataGTACAACGTCAGGTTTACCA (SEQ ID NO: 8)and the 3′ asRNA primer was GTAGCGCGGTGTATTATACC (SEQ ID NO: 9) (lowercase, T7 promoter sequence).

mRNAs were 5′-end-labeled using guanylyl transferase (Gibco/BRL), S-adenosyl methionine (Sigma), and α-^{32}P-GTP (3000 Ci/mmol; New England Nuclear) according to the manufacturer's directions. Radiolabeled RNAs were purified by poly(A) selection using the Poly(A) Tract III kit (Promega). Nonradioactive 7-methyl- guanosine- and adenosine-capped RNAs were synthesized in in vitro transcription reactions with a 5-fold excess of 7-methyl-G(5′)ppp(5′)G or A(5′)ppp(5′)G relative to GTP. Cap analogs were purchased from New England Biolabs.

ATP depletion and Protein Synthesis Inhibition

ATP was depleted by incubating the lysate for 10 minutes at 25°C with 2 mM glucose and 0.1 U/ml hexokinase (Sigma). Protein synthesis inhibitors were purchased from Sigma and dissolved in absolute ethanol as 250-fold concentrated

stocks. The final concentrations of inhibitors in the reaction were: anisomycin, 53 mg/ml; cycloheximide, 100 mg/ml; chloramphenicol, 100 mg/ml. Relative protein synthesis was determined by measuring the activity of Rr luciferase protein produced by translation of the Rr-luc mRNA in the RNAi reaction after 1 hour as described previously (Tuschl et al., Genes Dev., 13:3191-7 (1999)).

Analysis of dsRNA Processing

Internally α-^{32}P-ATP-labeled dsRNAs (505 bp Pp-luc or 501 Rr-luc) or 7-methyl-guanosine-capped Rr-luc antisense RNA (501 nt) were incubated at 5 nM final concentration in the presence or absence of unlabeled mRNAs in Drosophila lysate for 2 hours in standard conditions. Reactions were stopped by the addition of 2x proteinase K buffer and deproteinized as described previously (Tuschl et al., Genes Dev., 13:3191- 3197 (1999)). Products were analyzed by electrophoresis in 15% or 18% polyacrylamide sequencing gels. Length standards were generated by complete RNase T1 digestion of α-^{32}P-ATP-labeled 501 nt Rr-luc sense RNA and asRNA.

For analysis of mRNA cleavage, 5′-^{32}P-radiolabeled mRNA (described above) was incubated with dsRNA as described previously (Tuschl et al., Genes Dev., 13:3191- 3197 (1999)) and analyzed by electrophoresis in 5% (Figure 5B) and 6% (Figure 6C) polyacrylamide sequencing gels. Length standards included commercially available RNA size standards (FMC Bioproducts) radiolabeled with guanylyl transferase as described above and partial base hydrolysis and RNase T1 ladders generated from the 5′-radiolabeled mRNA.

Deamination Assay

Internally α-^{32}P-ATP-labeled dsRNAs (5 nM) were incubated in Drosophila lysate for 2 hours at standard conditions. After deproteinization, samples were run on 12% sequencing gels to separate full-length dsRNAs from the 21-23 nt products. RNAs were eluted from the gel slices in 0.3 M NaCl overnight, ethanol-precipitated, collected by centrifugation, and redissolved in 20 µl water. The RNA was hydrolyzed into nucleoside 5 -phosphates with nuclease P1 (10 µl reaction containing 8 µl RNA in water, 30 mM KOAc pH 5.3, 10 mM $ZnSO_4$, 10 µg or 3

units nuclease P1, 3 hours, 50° C). Samples (1 ml) were co-spotted with non-radioactive 5 -mononucleotides [0.05 O.D. units (A_{260}) of pA, pC, pG, pI, and pU] on cellulose HPTLC plates (EM Merck) and separated in the first dimension in isobutyric acid/25% ammonia/water (66/1/33, v/v/v) and in the second dimension in 0.1M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol (100/60/2, v/w/v; Silberklang et al., 1979). Migration of the non- radioactive internal standards was determined by UV-shadowing.

Results and Discussion

RNAi Requires ATP

As described in Example 1, Drosophila embryo lysates faithfully recapitulate RNAi (Tuschl et al., Genes Dev., 13:3191-7 (1999)). Previously, dsRNA-mediated gene silencing was monitored by measuring the synthesis of luciferase protein from the targeted mRNA. Thus, these RNAi reactions contained an ATP-regenerating system, needed for the efficient translation of the mRNA. To test if ATP was, in fact, required for RNAi, the lysates were depleted for ATP by treatment with hexokinase and glucose, which converts ATP to ADP, and RNAi was monitored directly by following the fate of ^{32}P-radiolabeled Renilla reniformis luciferase (Rr-luc) mRNA (Figure 6). Treatment with hexokinase and glucose reduced the endogenous ATP level in the lysate from 250 μM to below 10 μM. ATP regeneration required both exogenous creatine phosphate and creatine kinase, which acts to transfer a high-energy phosphate from creatine phosphate to ADP. When ATP-depleted extracts were supplemented with either creatine phosphate or creatine kinase separately, no RNAi was observed. Therefore, RNAi requires ATP in vitro. When ATP, creatine phosphate, and creatine kinase were all added together to reactions containing the ATP-depleted lysate, dsRNA-dependent degradation of the Rr-luc mRNA was restored (Figure 6). The addition of exogenous ATP was not required for efficient RNAi in the depleted lysate, provided that both creatine phosphate and creatine kinase were present, demonstrating that the endogenous concentration (250 mM) of adenosine nucleotide is sufficient to support RNAi. RNAi with a Photinus pyralis luciferase (Pp-luc) mRNA was also ATP-dependent.

The stability of the Rr-luc mRNA in the absence of Rr-dsRNA was reduced in ATP-depleted lysates relative to that observed when the energy regenerating system was included, but decay of the mRNA under these conditions did not display the rapid decay kinetics characteristic of RNAi in vitro, nor did it generate the stable mRNA cleavage products characteristic of dsRNA-directed RNAi. These experiments do not establish if the ATP requirement for RNAi is direct, implicating ATP in one or more steps in the RNAi mechanism, or indirect, reflecting a role for ATP in maintaining high concentrations of another nucleoside triphosphate in the lysate.

Translation Is Not Required for RNAi In Vitro

The requirement for ATP suggested that RNAi might be coupled to mRNA translation, a highly energy-dependent process. To test this possibility, various inhibitors of protein synthesis were added to the reaction by preparing a denaturing agarose-gel analysis of 5′-32P-radiolabeled Pp-luc mRNA after incubation for indicated times in a standard RNAi reaction with and without protein synthesis inhibitors. The eukaryotic translation inhibitors anisomycin, an inhibitor of initial peptide bond formation, cycloheximide, an inhibitor of peptide chain elongation, and puromycin, a tRNA mimic which causes premature termination of translation (Cundliffe, Antibiotic Inhibitors of Ribosome Function. In The Molecular Basis of Antibiotic Action, E. Gale, E. Cundliffe, P. Reynolds, M. Richmond and M. Warning, eds. (New York: Wiley), pp. 402-547. (1981)) were tested. Each of these inhibitors reduced protein synthesis in the Drosophila lysate by more than 1,900-fold (Figure 7A). In contrast, chloramphenicol, an inhibitor of Drosophila mitochondrial protein synthesis (Page and Orr-Weaver, Dev. Biol., 183:195-207 (1997)), had no effect on translation in the lysates (Figure 7A). Despite the presence of anisomycin, cycloheximide, or chloramphenicol, RNAi proceeded at normal efficiency. Puromycin also did not perturb efficient RNAi. Thus, protein synthesis is not required for RNAi in vitro.

Translational initiation is an ATP-dependent process that involves recognition of the 7-methyl guanosine cap of the mRNA (Kozak, Gene, 234:187-208

(1999); Merrick and Hershey, The Pathway and Mechanism of Eukaryotic Protein Synthesis. In Translational Control, J. Hershey, M. Mathews and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 31-69 (1996)). The Drosophila lysate used to support RNAi in vitro also recapitulates the cap-dependence of translation; Pp-luc mRNA with a 7-methyl-guanosine cap was translated greater than ten-fold more efficiently than was the same mRNA with an A(5′)ppp(5′)G cap (Figure 7B). Both RNAs were equally stable in the Drosophila lysate, showing that this difference in efficiency cannot be merely explained by more rapid decay of the mRNA with an adenosine cap (see also Gebauer et al., EMBO J., 18:6146-54 (1999)). Although the translational machinery can discriminate between Pp-luc mRNAs with 7- methyl-guanosine and adenosine caps, the two mRNAs were equally susceptible to RNAi in the presence of Pp-dsRNA (Figure 7C). These results suggest that steps in cap recognition are not involved in RNAi.

dsRNA Is Processed to 21-23 nt Species

RNAs 25 nt in length are generated from both the sense and anti-sense strands of genes undergoing post-transcriptional gene silencing in plants (Hamilton and Baulcombe, Science, 286:950-2 (1999)). Denaturing acrylamide-gel analysis of the products formed in a two-hour incubation of uniformly ^{32}P-radiolabeled dsRNAs and capped asRNA in lysate under standard RNAi conditions, in the presence or absence of target mRNAs. It was found that dsRNA is also processed to small RNA fragments. When incubated in lysate, approximately 15% of the input radioactivity of both the 501 bp Rr-dsRNA and the 505 bp Pp-dsRNA appeared in 21 to 23 nt RNA fragments. Because the dsRNAs are more than 500 bp in length, the 15% yield of fragments implies that multiple 21-23 nt RNAs are produced from each full-length dsRNA molecule. No other stable products were detected. The small RNA species were produced from dsRNAs in which both strands were uniformly ^{32}P-radiolabeled. Formation of the 21-23 nt RNAs from the dsRNA did not require the presence of the corresponding mRNA, demonstrating that the small RNA species is generated by processing of the dsRNA, rather than as a product of

dsRNA-targeted mRNA degradation. It was noted that 22 nucleotides corresponds to two turns of an A-form RNA-RNA helix.

When dsRNAs radiolabeled within either the sense or the anti-sense strand were incubated with lysate in a standard RNAi reaction, 21-23 nt RNAs were generated with comparable efficiency. These data support the idea that the 21-23 nt RNAs are generated by symmetric processing of the dsRNA. A variety of data support the idea that the 21-23 nt RNA is efficiently generated only from dsRNA and is not the consequence of an interaction between single-stranded RNA and the dsRNA. First, a ^{32}P-radiolabeled 505 nt Pp-luc sense RNA or asRNA was not efficiently converted to the 21-23 nt product when it was incubated with 5 nM nonradioactive 505 bp Pp- dsRNA. Second, in the absence of mRNA, a 501 nt 7-methyl-guanosine-capped Rr- asRNA produced only a barely detectable amount of 21-23 nt RNA (capped single- stranded RNAs are as stable in the lysate as dsRNA, Tuschl et al., Genes Dev., 13:3191- 7 (1999)), probably due to a small amount of dsRNA contaminating the anti-sense preparation. However, when Rr-luc mRNA was included in the reaction with the ^{32}P- radiolabeled, capped Rr-asRNA, a small amount of 21-23 nt product was generated, corresponding to 4% of the amount of 21-23 nt RNA produced from an equimolar amount of Rr-dsRNA. This result is unlikely to reflect the presence of contaminating dsRNA in the Rr-asRNA preparation, since significantly more product was generated from the asRNA in the presence of the Rr-luc mRNA than in the absence. Instead, the data suggest that asRNA can interact with the complementary mRNA sequences to form dsRNA in the reaction and that the resulting dsRNA is subsequently processed to the small RNA species. Rr-asRNA can support a low level of bona fide RNAi in vitro (see below), consistent with this explanation.

It was next asked if production of the 21-23 nt RNAs from dsRNA required ATP. When the 505 bp Pp-dsRNA was incubated in a lysate depleted for ATP by treatment with hexokinase and glucose, 21-23 nt RNA was produced, albeit 6 times slower than when ATP was regenerated in the depleted lysate by the inclusion of creatine kinase and creatine phosphate. Therefore, ATP may not be required for production of the 21-23 nt RNA species, but may instead simply enhance its

formation. Alternatively, ATP may be required for processing of the dsRNA, but at a concentration less than that remaining after hexokinase treatment. The molecular basis for the slower mobility of the small RNA fragments generated in the ATP-depleted lysate is not understood.

Wagner and Sun (Wagner and Sun, Nature, 391:744-745 (1998)) and Sharp (Sharp, Genes Dev., 13:139-41 (1999)) have speculated that the requirement for dsRNA in gene silencing by RNAi reflects the involvement of a dsRNA-specific adenosine deaminase in the process. dsRNA adenosine deaminases unwind dsRNA by converting adenosine to inosine, which does not base-pair with uracil. dsRNA adenosine deaminases function in the post-transcriptional editing of mRNA (for review see Bass, Trends Biochem. Sci., 22:157-62 (1997)). To test for the involvement of dsRNA adenosine deaminase in RNAi, the degree of conversion of adenosine to inosine in the 501 bp Rr-luc and 505 bp Pp-luc dsRNAs after incubation with Drosophila embryo lysate in a standard in vitro RNAi reaction was examined. Adenosine deamination in full-length dsRNA and the 21-23 nt RNA species was assessed by two-dimensional thin-layer chromatography. Inorganic phosphate (P_i,) was produced by the degradation of mononucleotides by phosphatases that contaminate commercially available nuclease P1 (Auxilien et al., J. Mol. Biol., 262:437-458 (1996)). The degree of adenosine deamination in the 21-23 nt species was also determined. The full-length dsRNA radiolabeled with [^{32}P]-adenosine was incubated in the lysate, and both the full-length dsRNA and the 21-23 nt RNA products were purified from a denaturing acrylamide gel, cleaved to mononucleotides with nuclease P1, and analyzed by two-dimensional thin- layer chromatography.

A significant fraction of the adenosines in the full-length dsRNA were converted to inosine after 2 hours (3.1% and 5.6% conversion for Pp-luc and Rr-luc dsRNAs, respectively). In contrast, only 0.4% (Pp-dsRNA) or 0.7% (Rr-dsRNA) of the adenosines in the 21-23 nt species were deaminated. These data imply that fewer than 1 in 27 molecules of the 21-23 nt RNA species contain an inosine. Therefore, it is unlikely that dsRNA-dependent adenosine deamination within the 21-23 nt species is required for its production.

asRNA Generates a Small Amount of RNAi in vitro

When mRNA was ^{32}P-radiolabeled within the 5′-7-methyl-guanosine cap, stable 5′ decay products accumulated during the RNAi reaction. Such stable 5′decay products were observed for both the Pp-luc and Rr-luc mRNAs when they were incubated with their cognate dsRNAs. Previously, it was reported that efficient RNAi does not occur when asRNA is used in place of dsRNA (Tuschl et al., Genes Dev., 13:3191-7 (1999)). Nevertheless, mRNA was measurably less stable when incubated with asRNA than with buffer (Figures 8A and 8B). This was particularly evident for the Rr-luc mRNA: approximately 90% of the RNA remained intact after a 3-hour incubation in lysate, but only 50% when asRNA was added. Less than 5% remained when dsRNA was added. Interestingly, the decrease in mRNA stability caused by asRNA was accompanied by the formation of a small amount of the stable 5′-decay products characteristic of the RNAi reaction with dsRNA. This finding parallels the observation that a small amount of 21- 23 nt product formed from the asRNA when it was incubated with the mRNA (see above) and lends strength to the idea that asRNA can enter the RNAi pathway, albeit inefficiently.

mRNA Cleavage Sites Are Determined by the Sequence of the dsRNA

The sites of mRNA cleavage were examined using three different dsRNAs, 'A,' 'B,' and 'C,' displaced along the Rr-luc sequence by approximately 100 nts. Denaturing acrylamide-gel analysis of the stable, 5′-cleavage products produced after incubation of the Rr-luc mRNA for the indicated times with each of the three dsRNAs, 'A,' 'B,' and 'C,' or with buffer (Ø) was performed. The positions of these relative to the Rr-luc mRNA sequence are shown in Figure 9. Each of the three dsRNAs was incubated in a standard RNAi reaction with Rr-luc mRNA ^{32}P-radiolabeled within the 5′-cap. In the absence of dsRNA, no stable 5′-cleavage products were detected for the mRNA, even after 3 hours of incubation in lysate. In contrast, after a 20-minute incubation, each of the three dsRNAs produced a ladder of bands corresponding to a set of mRNA cleavage products characteristic for that particular dsRNA. For each dsRNA, the stable, 5′ mRNA cleavage products were restricted to the region of the Rr-luc mRNA that corresponded to the dsRNA

(Figures 9 and 10). For dsRNA 'A,' the lengths of the 5′- cleavage products ranged from 236 to just under ~750 nt; dsRNA 'A' spans nucleotides 233 to 729 of the Rr-luc mRNA. Incubation of the mRNA with dsRNA 'B' produced mRNA 5′-cleavage products ranging in length from 150 to ~600 nt; dsRNA 'B' spans nucleotides 143 to 644 of the mRNA. Finally, dsRNA 'C' produced mRNA cleavage products from 66 to ~500 nt in length. This dsRNA spans nucleotides 50 to 569 of the Rr-luc mRNA. Therefore, the dsRNA not only provides specificity for the RNAi reaction, selecting which mRNA from the total cellular mRNA pool will be degraded, but also determines the precise positions of cleavage along the mRNA sequence.

The mRNA Is Cleaved at 21-23 Nucleotide Intervals

To gain further insight into the mechanism of RNAi, the positions of several mRNA cleavage sites for each of the three dsRNAs were mapped (Figure 10). High resolution denaturing acrylamide-gel analysis of a subset of the 5′-cleavage products described above was performed. Remarkably, most of the cleavages occurred at 21-23 nt intervals (Figure 10). This spacing is especially striking in light of our observation that the dsRNA is processed to a 21-23 nt RNA species and the finding of Hamilton and Baulcombe that a 25 nt RNA correlates with post-transcriptional gene silencing in plants (Hamilton and Baulcombe, Science, 286:950-2 (1999)). Of the 16 cleavage sites we mapped (2 for dsRNA 'A,' 5 for dsRNA 'B,' and 9 for dsRNA 'C'), all but two reflect the 21-23 nt interval. One of the two exceptional cleavages was a weak cleavage site produced by dsRNA 'C' (indicated by an open blue circle in Figure 10). This cleavage occurred 32 nt 5′ to the next cleavage site. The other exception is particularly intriguing. After four cleavages spaced 21-23 nt apart, dsRNA 'C' caused cleavage of the mRNA just nine nt 3′ to the previous cleavage site (red arrowhead in Figure 10). This cleavage occurred in a run of seven uracil residues and appears to "reset" the ruler for cleavage; the next cleavage site was 21-23 nt 3′ to the exceptional site. The three subsequent cleavage sites that we mapped were also spaced 21-23 nt apart. Curiously, of the sixteen cleavage sites caused by the three different dsRNAs, fourteen occur at uracil residues. The

significance of this finding is not understood, but it suggests that mRNA cleavage is determined by a process which measures 21-23 nt intervals and which has a sequence preference for cleavage at uracil. Results show that the 21-23 nt RNA species produced by incubation of ~500 bp dsRNA in the lysate caused sequence-specific interference in vitro when isolated from an acrylamide gel and added to a new RNAi reaction in place of the full-length dsRNA.

A Model for dsRNA-directed mRNA Cleavage

Without wishing to be bound by theory, the biochemical data described herein, together with recent genetic experiments in C. elegans and Neurospora (Cogoni and Macino, Nature, 399:166-9 (1999); Grishok et al., Science, 287: 2494-7 (2000); Ketting et al., Cell, 99:133-41 (1999); Tabara et al., Cell, 99:123-32 (1999)), suggest a model for how dsRNA targets mRNA for destruction (Figure 11). In this model, the dsRNA is first cleaved to 21-23 nt long fragments in a process likely to involve genes such as the C. elegans loci rde-1 and rde-4. The resulting fragments, probably as short asRNAs bound by RNAi-specific proteins, would then pair with the mRNA and recruit a nuclease that cleaves the mRNA. Alternatively, strand exchange could occur in a protein-RNA complex that transiently holds a 21-23 nt dsRNA fragment close to the mRNA. Separation of the two strands of the dsRNA following fragmentation might be assisted by an ATP-dependent RNA helicase, explaining the observed ATP enhancement of 21-23 nt RNA production.

It is likely that each small RNA fragment produces one, or at most two, cleavages in the mRNA, perhaps at the 5′ or 3′ ends of the 21-23 nt fragment. The small RNAs may be amplified by an RNA-directed RNA polymerase such as that encoded by the ego-1 gene in C. elegans (Smardon et al., Current Biology, 10:169-178 (2000)) or the qde-1 gene in Neurospora (Cogoni and Macino, Nature, 399:166-9 (1999)), producing long-lasting post-transcriptional gene silencing in the absence of the dsRNA that initiated the RNAi effect. Heritable RNAi in C. elegans requires the rde-1 and rde-4 genes to initiate, but not to persist in subsequent generations. The rde-2, rde- 3, and mut-7 genes in C. elegans are required in the tissue where RNAi occurs, but are not required for initiation of heritable RNAi

(Grishok et al., Science, in press 2000). These 'effector' genes (Grishok et al., Science, in press 2000) are likely to encode proteins functioning in the actual selection of mRNA targets and in their subsequent cleavage. ATP may be required at any of a number of steps during RNAi, including complex formation on the dsRNA, strand dissociation during or after dsRNA cleavage, pairing of the 21-23 nt RNAs with the target mRNA, mRNA cleavage, and recycling of the targeting complex. Testing these ideas with the in vitro RNAi system will be an important challenge for the future. Some genes involved in RNAi are also important for transposon silencing and co-suppresion. Co-suppression is a broad biological phenomenon spanning plants, insects and perhaps humans. The most likely mechanism in Drosophila melanogaster is transcriptional silencing (Pal-Bhanra et al, Cell 99: 35-36. Thus, 21-23 nt fragments are likely to be involved in transcriptional control, as well as in post-transcriptional cotrol.

Example 3 Isolated 21-23 mers caused sequence-specific interference when added to a new RNAi reaction

Isolation of 21-23 nt fragments from incubation reaction of 500 bp dsRNA in lysate.

Double-stranded RNA (500 bp from) was incubated at 10 nM concentration in Drosophila embryo lysate for 3 h at 25° C under standard conditions as described herein. After deproteinization of the sample, the 21-23 nt reaction products were separated from unprocessed dsRNA by denaturing polyacrylamide (15%) gel electrophoresis. For detection of the non-radiolabeled 21-23 nt fragments, an incubation reaction with radiolabeled dsRNA was loaded in a separate lane of the same gel. Gel slices containing the non-radioactive 21-23 nt fragments were cut out and the 21-23 nt fragments were eluted from the gel slices at 4° C overnight in 0.4 ml 0.3 M NaCl. The RNA was recovered from the supernatant by ethanol precipitation and centrifugation. The RNA pellet was dissolved in 10 µl of lysis buffer. As control, gel slices slightly above and below the 21-23 nt band were also cut out and subjected to the same elution and precipitation procedures. Also, a non-incubated dsRNA loaded on the 15% gel and a gel slice corresponding to 21-23 nt fragments was cut out and eluted. All pellets from the control experiments were

dissolved in 10 µl lysis buffer. The losses of RNA during recovery from gel slices by elution are approx. 50%.

Incubation of purified 21-23 nt fragments in a translation-based RNAi assay

1 µl of the eluted 21-23 mer or control RNA solution was used for a standard 10 µl RNAi incubation reaction (see above). The 21-23 mers were preincubated in the lysate containing reaction mixture for 10 or 30 min before the addition of the target and control mRNA. During pre-incubation, proteins involved in RNA interference may re- associate with the 21-23 mers due to a specific signal present on these RNAs. The incubation was continued for another hour to allow translation of the target and control mRNAs. The reaction was quenched by the addition of passive lysis buffer (Promega), and luciferase activity was measured. The RNA interference is the expressed as the ratio of target to control luciferase activity normalized by an RNA-free buffer control. Specific suppression of the target gene was observed with either 10 or 30 minutes pre- incubation. The suppression was reproducible and reduced the relative ratio of target to control by 2-3 fold. None of the RNA fragments isolated as controls showed specific interference. For comparison, incubation of 5 nM 500 bp dsRNA (10 min pre- incubation) affects the relative ratio of control to target gene approx. 30-fold.

Stability of isolated 21-23 nt fragments in a new lysate incubation reaction.

Consistent with the observation of RNAi mediated by purified 21-23 nt RNA fragment, it was found that 35% of the input 21-23 nt RNA persists for more than 3 h in such an incubation reaction. This suggests that cellular factors associate with the deproteinized 21-23 nt fragments and reconstitute a functional mRNA-degrading particle. Signals connected with these 21-23 nt fragments, or their possible double stranded nature or specific lengths are likely responsible for this observation. The 21-23 nt fragments have a terminal 3' hydroxyl group, as evidenced by altered mobility on a sequencing gel following periodate treatment and beta-elimination.

Example 4 21-23-mers purified by non-denaturing methods caused sequence-specific interference when added to a new RNAi reaction.

Fifty nanomolar double-stranded RNA (501 bp Rr-luc dsRNA, as described in example 1) was incubated in a 1 ml in vitro reaction with lysate at 25°C (see example 1). The reaction was then stopped by the addition of an equal volume of 2x PK buffer (see example 1) and proteinase K was added to a final concentration of 1.8 µg/µl. The reaction was incubated for an additional 1 h at 25°C, phenol extracted, and then the RNAs were precipitated with 3 volumes of ethanol. The ethanol precipitate was collected by centrifugation, and the pellet was resuspended in 100 µl of lysis buffer and applied to a Superdex HR 200 10/30 gel filtration column (Pharmacia) run in lysis buffer at 0.75 ml/min. 200 µl fractions were collected from the column. Twenty µl of 3 M sodium acetate and 20 µg glycogen was added to each fraction, and the RNA was recovered by precipitation with 3 volumes of ethanol. The precipitates were resuspended in 30 µl of lysis buffer. Column profiles following the fractionation of 32P-labeled input RNA are shown in Figure 13A.

One microliter of each resuspended fraction was tested in a 10 µl standard in vitro RNAi reaction (see example 1). This procedure yields a concentration of RNA in the in vitro RNAi reaction that is approximately equal to the concentration of that RNA species in the original reaction prior to loading on the column. The fractions were preincubated in the lysate containing reaction mixture for 30 min before the addition of 10 nM Rr-luc mRNA target and 10 nM Pp-luc control mRNA. During pre-incubation, proteins involved in RNA interference may re-associate with the 21-23-mers due to a specific signal present on these RNAs. The incubation was continued for another three hours to allow translation of the target and control mRNAs. The reaction was quenched by the addition of passive lysis buffer (Promega), and luciferase activity was measured. The suppression of Rr-luc mRNA target expression by the purified 21-23 nt fragments was reproducible and reduced the relative ratio of target to control by >30-fold, an amount comparable to a 50 nM 500 bp dsRNA control. Suppression of target mRNA expression was specific: little or no effect on the expression of the Pp-luc mRNA control was observed.

The data show that the both the fractions containing uncleaved dsRNA (fractions 3 - 5) or long, partially cleaved dsRNA (fractions 7 - 13) and the fractions containing the fully processed 21-23 nt siRNAs (fractions 41 - 50) mediate effective RNA interference in vitro (Figure 13B). Suppression of target mRNA expression was specific: little or no effect on the expression of the Pp-luc mRNA control was observed (Figure 13C). These data, together with those in the earlier examples, demonstrate that the 21-23 nt siRNAs are (1) true intermediates in the RNAi pathway and (2) effective mediators of RNA interference in vitro.

Example 5 21-nucleotide siRNA duplexes mediate RNA interference in human tissue cultures

Methods

RNA preparation

21 nt RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified (Elbashir, S.M., Lendeckel, W. & Tuschl, T., *Genes & Dev. 15*, 188-200 (2001)), followed by Sep-Pak C18 cartridge (Waters, Milford, MA, USA) purification (Tuschl, t., *et al., Biochemistry, 32:*11658-11668 (1993)). The siRNA sequences targeting GL2 (Acc. X65324) and GL3 luciferase (Acc. U47296) corresponded to the coding regions 153-173 relative to the first nucleotide of the start codon, siRNAs targeting RL (Acc. AF025846) corresponded to region 119-129 after the start codon. Longer RNAs were transcribed with T7 RNA polymerase from PCR products, followed by gel and Sep-Pak purification. The 49 and 484 bp GL2 or GL3 dsRNAs corresponded to position 113-161 and 113-596, respectively, relative to the start of translation; the 50 and 501 bp RL dsRNAs corresponded to position 118-167 and 118-618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (Kehlenbach, R.H., *et al., J. Cell Biol., 141:*863-874 (1998)), whereby 50 and 501 bp hG dsRNA corresponded to position 118-167 and 118-618, respectively, to the start codon.

For annealing of siRNAs, 20 μM single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h at 37 °C. The 37 °C incubation step was extended overnight for the 50 and 500 bp dsRNAs, and these annealing reactions were performed at 8.4 μM and 0.84 μM strand concentrations, respectively.

Cell culture

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 25 °C. 293, NIH/3T3, HeLa S3, COS-7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were regularly passaged to maintain exponential growth. 24 h before transfection at approx. 80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1-3 × 10^5 cells/ml) and transferred to 24-well plates (500 μl/well). S2 cells were not trypsinized before splitting. Transfection was carried out with Lipofectamine 2000 reagent (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 μg pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 μg pRL-TK (Promega), and 0.28 μg siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 μl per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cell lines after co-transfection of 1.1 μg hGFP-encoding pAD3[22] and 0.28 μg invGL2 siRNA, and were 70-90%. Reporter plasmids were amplified in XL-1 Blue (Strategene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

Results

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded

RNA (dsRNA) homologous in sequence to the silenced gene (Fire, A., *Trends Genet., 15:*358-363 (1999); Sharp, P.A. & Zamore, P.D., *Science, 287:*2431-2433 (2000); Sijen, T. & Kooter, J.M., *Bioessays, 22:*520-531 (2000); Bass, B.L., *Cell, 101:*235-238 (2000); Hammond, S.M., *et al., Nat. Rev. Genet., 2:*110-119 (2001)). The mediators of sequence-specific mRNA degradation are 21 and 22 nt small interfering RNAs (siRNAs) generated by RNase III cleavage from longer dsRNAs[6-10] (Hamilton, A.J. &Baulcombe, D.C., *Science, 286:*950-952 (1999); Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Zamore, P.D., *et al., Cell, 101:*25-33 (2000); Bernstein, E., *et al., Naature, 409:*363-366 (2001); Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). As shown herein, 21 nt siRNA duplexes are able to specifically suppress reporter gene expression in multiple mammalian tissue cultures, including human embryonic kidney (293) and HeLa cells. In contrast to 50 or 500 bp dsRNAs, siRNAs do not activate the interferon response. These results indicate that siRNA duplexes are a general tool for sequence-specific inactivation of gene function in mammalian cells.

Base-paired 21 and 22 nt siRNAs with overhanging 3' ends mediate efficient sequence-specific mRNA degradation in lysates prepared from *D. melanogaster* embryos (Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). To test whether siRNAs are also capable of mediating RNAi in tissue culture, 21 nt siRNA duplexes with symmetric 2 nt 3' overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases (Figures 14A, 14B) were constructed. The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL, into *D. melanogaster Schneider S2* cells or mammalian cells using cationic liposomes. Luciferase activities were determined 20 h after transfection. In all cell lines tested, specific reduction of the expression of the reporter genes in the presence of cognate siRNA duplexes was observed (Figures 15A-15J). Remarkably, the absolute luciferase expression levels were unaffected by non-cognate siRNAs, indicating the absence of harmful side effects by 21 nt RNA duplexes (e.g. Figures 16A-16D, for HeLa cells). In *D. melanogaster* S2 cells (Figures 15A, 15B), the specific inhibition of luciferases was complete, and similar to results previously

obtained for longer dsRNAs (Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Caplen, N.J., *et al., Gene, 252:*95-105 (2000); Clemens, M & Williams, B., *Cell, 13:*565-572 (1978); Ui-Tei, K., *et al., FEBS Letters, 479:*79-82 (2000)). In mammalian cells, where the reporter genes were 50- to 100-fold stronger expressed, the specific suppression was less complete (Figures 15C-15J). GL2 expression was reduced 3- to 12-fold, GL3 expression 9- to 25-fold, and RL expression 1- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (Figures 15I, 15J). It is likely that the lack of reduction of RL expression in 293 cells is due to its 5- to 20-fold higher expression compared to any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

The 2 nt 3' overhang in all siRNA duplexes, except for uGL2, was composed of (2'-deoxy) thymidine. Substitution of uridine by thymidine in the 3' overhang was well tolerated in the *D. melanogaster* in vitro system, and the sequence of the overhang was uncritical for target recognition (Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). The thymidine overhang was chosen, because it is supposed to enhance nuclease resistance of siRNAs in the tissue culture medium and within transfected cells. Indeed, the thymidine-modified GL2 siRNA was slightly more potent than the unmodified uGL2 siRNA in all cell lines tested (Figures 15A, 15C, 15E, 15G, 15I). It is conceivable that further modifications of the 3' overhanging nucleotides will provide additional benefits to the delivery and stability of siRNA duplexes.

In co-transfection experiments, 25 nM siRNA duplexes with respect to the final volume of tissue culture medium were used (Figures 15A-15J, 16A-16F). Increasing the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies due to competition for liposome encapsulation between plasmid DNA and siRNA. Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect, even though the

siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing, and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene targeting experiments.

In order to monitor the effect of longer dsRNAs on mammalian cells, 50 and 500 bp dsRNAs cognate to the reporter genes were prepared. As non-specific control, dsRNAs from humanized GFP (hG) (Kehlenbach, R.H., *et al., J. Cell Biol., 141:*863-874 (1998)) was used. When dsRNAs were co-transfected, in identical amounts (not concentrations) to the siRNA duplexes, the reporter gene expression was strongly and unspecifically reduced. This effect is illustrated for HeLa cells as a representative example (Figures 16A-16D). The absolute luciferase activities were decreased unspecifically 10- to 20-fold by 50 bp dsRNA, and 20- to 200-fold by 500 bp dsRNA co-transfection, respectively. Similar unspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold unspecific reduction was observed only for 500 bp dsRNAs. Unspecific reduction in reporter gene expression by dsRNA > 30 bp was expected as part of the interferon response (Matthews, M., Interactions between viruses and the cellular machinery for protein synthesis in *Translational Control* (eds., Hershey, J., Matthews,M. & Sonenberg, N.) 505-548 (Cold Spring Harbor Laboratory Press, Plainview, NY; 1996); Kumar, M. & Carmichael, G.G., *Microbiol. Mol. Biol. Rev., 62:*1415-1434 (1998); Stark, G.R., *et al., Annu. Rev. Biochem., 67:*227-264 (1998)). Surprisingly, despite the strong unspecific decrease in reporter gene expression, additional sequence-specific, dsRNA-mediated silencing were reproducibly detected. The specific silencing effects, however, were only apparent when the relative reporter gene activities were normalized to the hG dsRNA controls (Figures 16E, 16F). A 2- to 10-fold specific reduction in response to cognate dsRNA was observed, also in the other three mammalian cell lines tested. Specific silencing effects with dsRNAs (356-1662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments (Ui-Tei, K., *et al., FEBS Letters, 479:*79-82 (2000)). Also, CHO-K1

cells appear to be deficient in the interferon response. In another report, 293, NIH/3T3, and BHK-21 cells were tested for RNAi using luciferase/lacZ reporter combinations and 829 bp specific lacZ or 717 bp unspecific GFP dsRNA (Caplen, N.J., *et al., Gene, 252:*95-105 (2000)). The failure of detecting RNAi in this case is likely due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, the results described herein indicate that RNAi is active in mammalian cells, but that the silencing effect is difficult to detect if the interferon system is activated by dsRNA >30 bp.

The mechanism of the 21 nt siRNA-mediated interference process in mammalian cells remains to be uncovered, and silencing may occur post-transcriptional and/or transcriptional. In *D. melanogaster* lysate, siRNA duplexes mediate post-transcriptional gene silencing by reconstitution of a siRNA-protein complexes (siRNPs), which are guiding mRNA recognition and targeted cleavage (Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Zamore, P.D., *et al., Cell, 101:*25-33 (2000); Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). In plants, dsRNA-mediated post-transcriptional silencing has also been linked to RNA-directed DNA methylation, which may also be directed by 21 nt siRNAs (Wassenegger, M., *Plant Mol. Biol, 43:*203-220 (2000); Finnegan, E.J., *et al., Curr. Biol., 11:*R99-R102 (2000)). Methylation of promoter regions can lead to transcriptional silencing (Metter, M.F., *et al., EMBO J., 19:*5194-5201 (2000)), but methylation in coding sequences must not (Wang, M.-B., *RNA, 7:*16-28 (2001)). DNA methylation and transcriptional silencing in mammals are well-documented processes (Kass, S.U., *et al., Trends Genet., 13:*444-449 (1997); Razin, A., *EMBO J, 17:*4905-4908 (1998)), yet they have not been linked to post-transcriptional silencing. Methylation in mammals is predominantly directed towards CpG residues. Because there is no CpG in the RL siRNA, but RL siRNA mediates specific silencing in mammalian tissue culture, it is unlikely that DNA methylation is critical for our observed silencing process. In summary, described herein, is siRNA-mediated gene silencing in mammalian cells. The use of 21 nt siRNAs holds great promise for inactivation of gene function in human tissue culture and the development of gene-specific therapeutics.

While this invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims

CLAIMS

What is claimed is:

1. Isolated RNA of from about 21 to about 23 nucleotides that mediates RNA interference of an mRNA to which it corresponds.

2. Isolated RNA of claim 1 that comprises a terminal 3' hydroxyl group.

3. Isolated RNA of claim 1 which is chemically synthesized RNA or an analog of a naturally occurring RNA.

4. An analog of isolated RNA of claim 1, wherein the analog differs from the RNA of claim 1 by the addition, deletion, substitution or alteration of one or more nucleotides.

5. Isolated RNA of from about 21 to about 23 nucleotides that inactivates a corresponding gene by transcriptional silencing.

6. A soluble extract that mediates RNA interference.

7. The soluble extract of Claim 6, wherein the extract is derived from Drosophila embryos.

8. The soluble extract of Claim 7 wherein the extract is derived from syncytial blastoderm Drosophila embryos.

9. A method of producing RNA of from about 21 to about 23 nucleotides in length comprising:

(a) combining double-stranded RNA with a soluble extract that mediates RNA interference, thereby producing a combination; and

(b) maintaining the combination of a) under conditions in which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides in length.

10. The method of Claim 9, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

11. The method of Claim 9 further comprising isolating the RNA of from about 21 to about 23 nucleotides from the combination.

12. RNA of about 21 to about 23 nucleotides produced by the method of Claim 9.

13. A method of producing RNA of from about 21 to about 23 nucleotides in length that mediates RNA interference of mRNA of a gene to be degraded, comprising:

(a) combining double-stranded RNA that corresponds to a sequence of the gene to be degraded with a soluble extract that mediates RNA interference, thereby producing a combination; and

(b) maintaining the combination of (a) under conditions under which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides that mediates RNA interference of the mRNA of the gene to be degraded, thereby producing RNA of from about 21 to about 23 nucleotides that mediates RNA interference of the mRNA.

14. The method of Claim 13, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

15. The method of Claim 13 further comprising isolating RNA of from about 21 to about 23 nucleotides from the combination.

16. Isolated RNA of from about 21 to about 23 nucleotides produced by the method of Claim 15.

17. A method of mediating RNA interference of mRNA of a gene in a cell or organism comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into the cell or organism;

(b) maintaining the cell or organism produced in (a) under conditions under which degradation of the mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism.

18. The method of Claim17 wherein the RNA of (a) is a chemically synthesized RNA or an analog of naturally occurring RNA.

19. The method of Claim 17, wherein the gene encodes a cellular mRNA or a viral mRNA.

20. A method of mediating RNA interference of mRNA of a gene in a cell or organism in which RNA interference occurs, comprising:

(a) combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract that mediates RNA interference, thereby producing a combination;

(b) maintaining the combination produced in (a) under conditions under which the double- stranded RNA is processed to RNA of from about 21 to about 23 nucleotides, thereby producing RNA of from about 21 to about 23 nucleotides;

(c) isolating RNA of from about 21 to about 23 nucleotides produced in (b);

(d) introducing RNA isolated in (c) into the cell or organism; and

(e) maintaining the cell or organism produced in (d) under conditions under which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism.

21. The method of Claim 20, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

22. The method of Claim 20, wherein the RNA is isolated using gel electrophoresis.

23. A method of mediating RNA interference of mRNA of a gene in a cell or organism in which RNA interference occurs, comprising: (a) introducing into the cell or organism RNA of from about 21 to about 23 nucleotides that mediates RNA interference of mRNA of the gene, thereby producing a cell or organism that contains the RNA and (b) maintaining the cell or organism that contains the RNA under conditions under which RNA interference occurs, thereby mediating RNA interference of mRNA of the gene in the cell or organism.

24. The method of claim 23, wherein the RNA of from about 21 to about 23 nucleotides is chemically synthesized RNA or an analog of RNA that mediates RNA interference.

25. The method of Claim 23, wherein the gene encodes a cellular mRNA or a viral mRNA.

26. A knockdown cell or organism generated by the method of claim 23.

27. The knockdown cell or organism of claim 26, wherein the cell or organism mimics a disease.

28. A method of examining the function of a gene in a cell or organism comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides that targets mRNA of the gene for degradation into the cell or organism, thereby producing a test cell or test organism;

(b) maintaining the test cell or test organism under conditions under which degradation of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is degraded; and

(c) observing the phenotype of the test cell or test organism produced in (b) and, optionally, comparing the phenotype observed to that of an appropriate control cell or control organism, thereby providing information about the function of the gene.

29. The method of Claim 28 wherein the RNA introduced in (a) is chemically synthesized or an analog of RNA that mediates RNA interference.

30. A method of examining the function of a gene in a cell or organism comprising

(a) combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract that mediates RNA interference, thereby producing a combination;

(b) maintaining the combination produced in (a) under conditions under which the double- stranded RNA is processed to RNA of about 21 to about 23 nucleotides, whereby RNA of about 21 to about 23 nucleotides is produced;

(c) isolating RNA of about 21 to about 23 nucleotides produced in (b);

(d) introducing the RNA isolated in (c) into the cell or organism, thereby producing a test cell or test organism;

(e) maintaining the test cell or test organism under conditions under which degradation of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is degraded; and

(f) observing the phenotype of the test cell or test organism produced in (e) and, optionally, comparing the phenotype observed to that of an appropriate control, thereby providing information about the function of the gene.

31. The method of claim 30, wherein the RNA comprises a terminal 3' hydroxyl group.

32. The method of claim 30, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

33. The method of claim 30, wherein the RNA is isolated using gel electrophoresis.

34. A composition comprising biochemical components of a Drosophila cell that process dsRNA to RNA of about 21 to about 23 nucleotides and a suitable carrier.

35. A composition comprising biochemical components of a cell that target mRNA of a gene to be degraded by RNA of about 21 to about 23 nucleotides.

36. A method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNA of from about 21 to about 23 nucleotides that targets the mRNA of the protein for degradation.

37. The method of claim 36 wherein RNA of from about 21 to about 23 nucleotides is chemically synthesized or an analog of RNA that mediates RNA interference.

38. A method of assessing whether an agent acts on a gene product comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into a cell or organism;

(b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs,

(c) introducing the agent into the cell or organism of (b); and

(d) determining whether the agent has an effect on the cell or organism, wherein if the agent has no effect on the cell or organism then the agent acts on the gene product or on a biological pathway that involves the gene product.

39. The method of claim 38, wherein the RNA of from about 21 to about 23 nucleotides is chemically synthesized or an analog of RNA that mediates RNA interference.

40. A method of assessing whether a gene product is a suitable target for drug discovery comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into a cell or organism;

(b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs resulting in decreased expression of the gene; and

(c) determining the effect of the decreased expression of the gene on the cell or organism, wherein if decreased expression has an effect, then the gene product is a target for drug discovery.

41. The method of claim 40, wherein the RNA of from about 21 to about 23 nucleotides is synthetic RNA or an analog of RNA that mediates RNA interference.

42. A gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

43. A pharmaceutical composition comprising RNA of from about 21 to about 23 nucleotides that mediates RNA interference and an appropriate carrier.

44. A method of producing knockdown cells, comprising introducing into cells in which a gene is to be knocked down RNA of about 21 to about 23 nt that targets the mRNA corresponding to the gene and maintaining the resulting cells under conditions under which RNAi occurs, resulting in degradation of the mRNA of the gene, thereby producing knockdown cells.

45. The method of claim 44, wherein the RNA of about 21 to about 23 nucleotides is synthetic RNA or an analog of RNA that mediates RNA interference.

46. A method of identifying target sites within mRNA that are efficiently cleaved by the RNAi process, comprising combining dsRNA corresponding to a sequence of a gene to be degraded, labeled mRNA corresponding to the gene and a soluble extract that mediates RNA interference, thereby producing a combination; maintaining the combination under conditions under which the dsRNA is degraded and identifying sites in the mRNA that are efficiently cleaved.

47. A method of identifying 21-23 nt RNAs that efficiently mediate RNAi, wherein said 21-23 nt RNAs span the target sites identified within the mRNA by the method of claim 46.

48. RNA of claim 16, isolated using gel electrophoresis.

49. RNA of claim 16, isolated using non-denaturing methods.

50. RNA of claim 16, isolated using non-denaturing column chromatography.

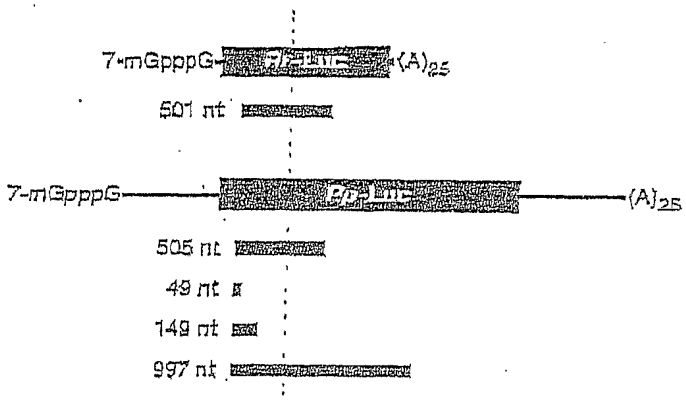


Figure 1

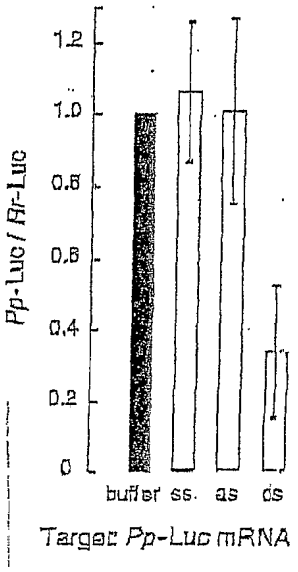


Figure 2A

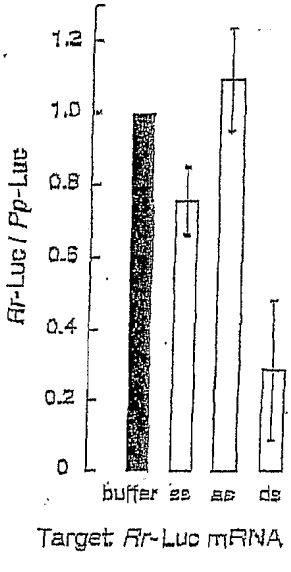


Figure 2B

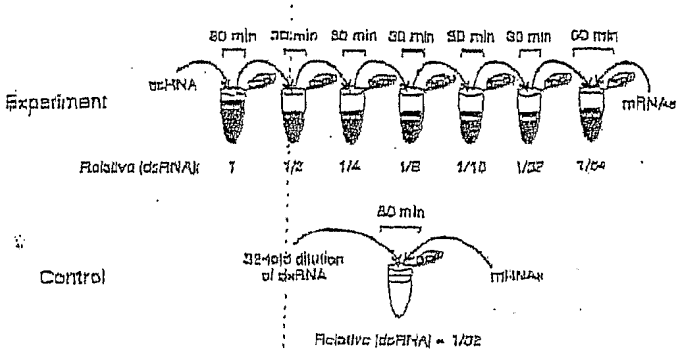


Figure 3A

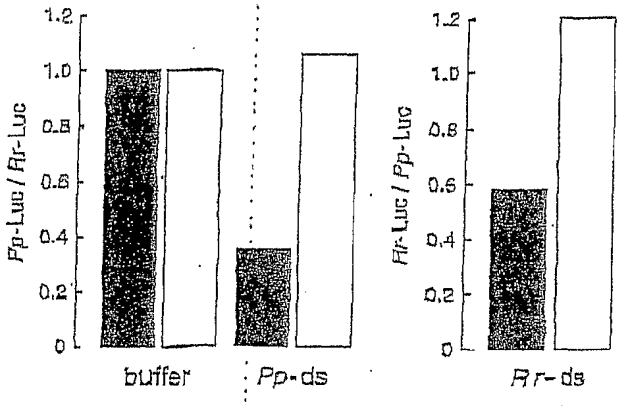


Figure 3B

Figure 3C

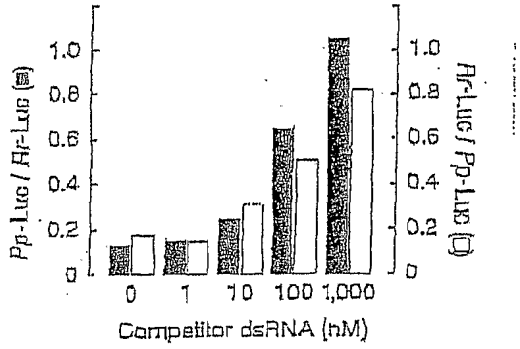


Figure 4

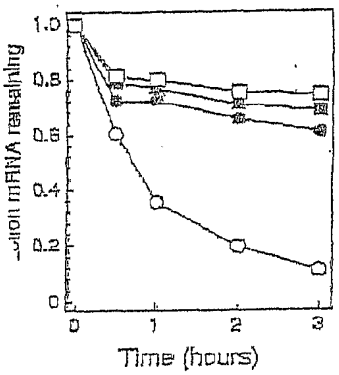


Figure 5A

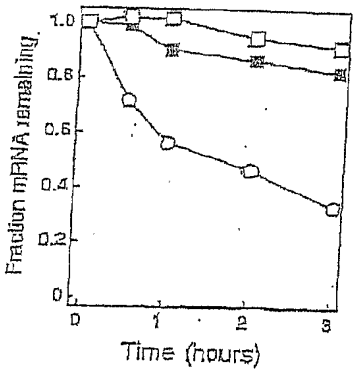


Figure 5B

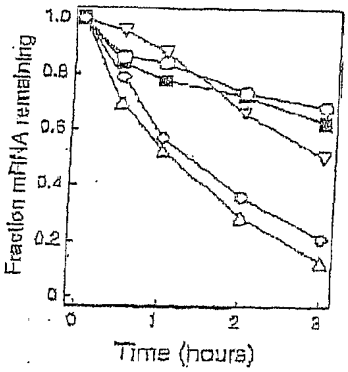


Figure 5C

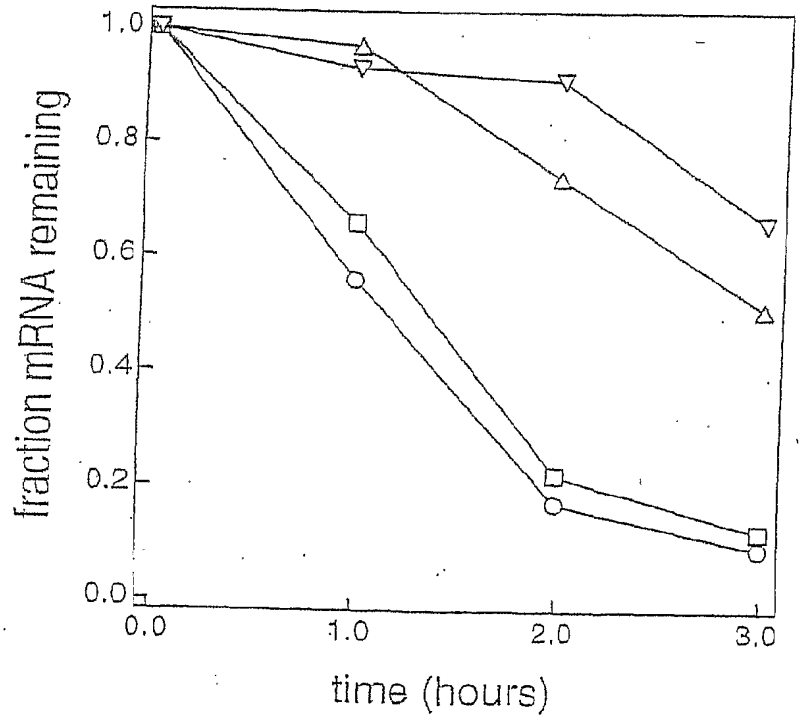


Figure 6

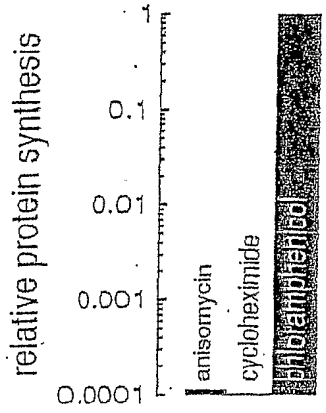


Figure 7A

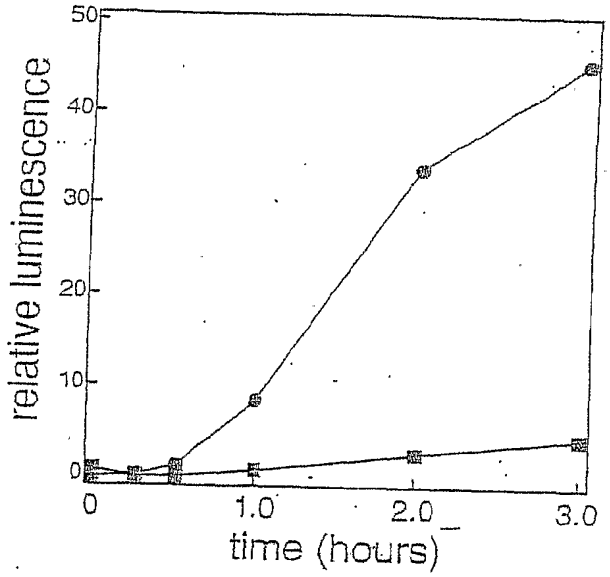


Figure 7B

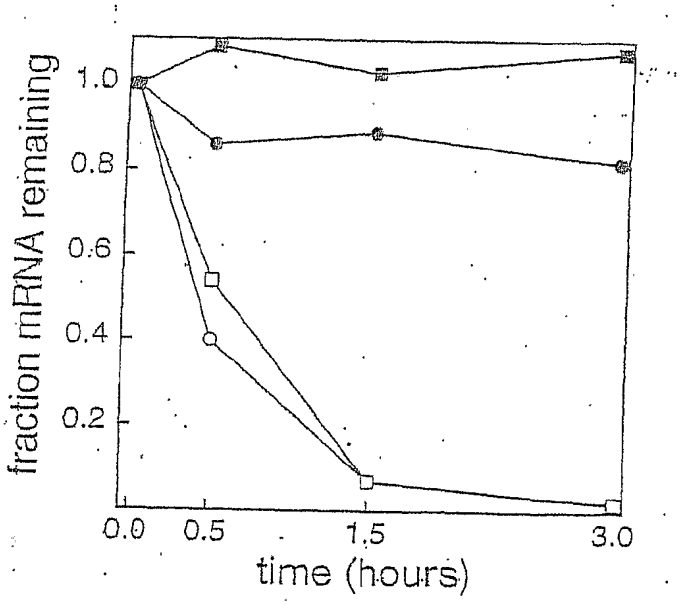


Figure 7C

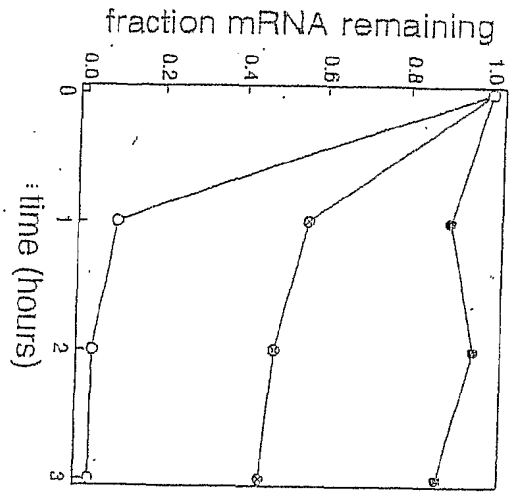


Figure 8A

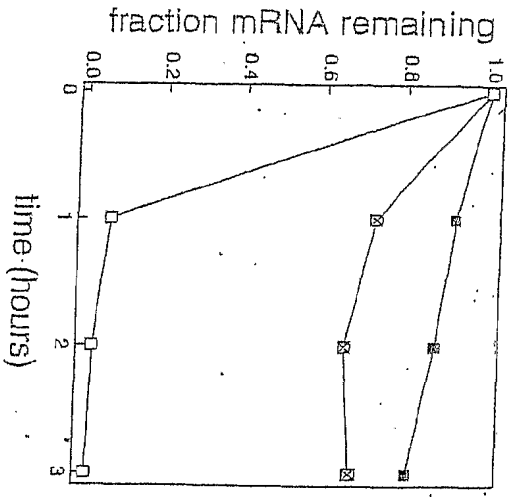


Figure 8B

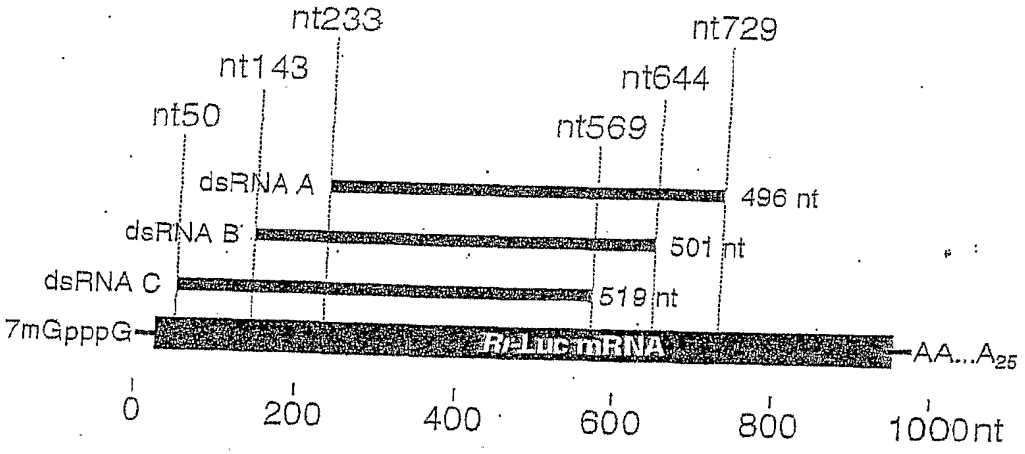
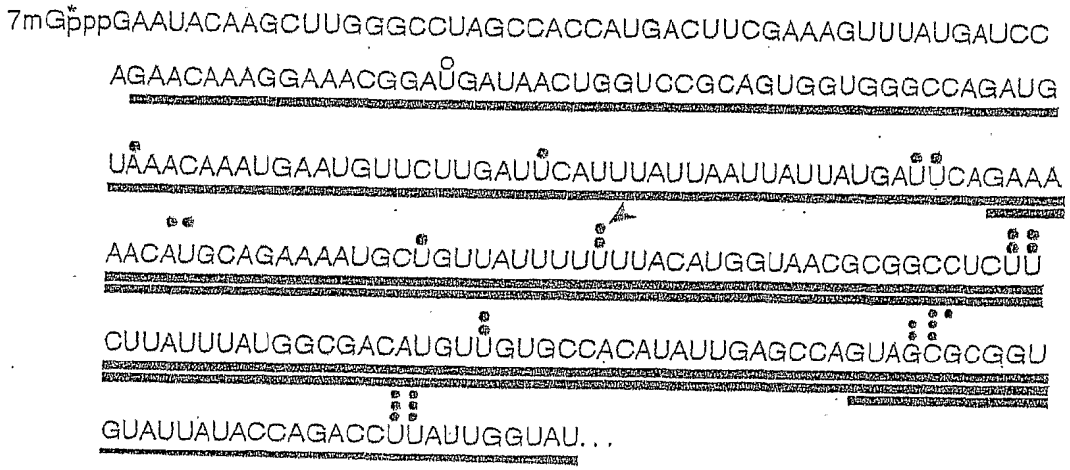


Figure 9

Figure 10



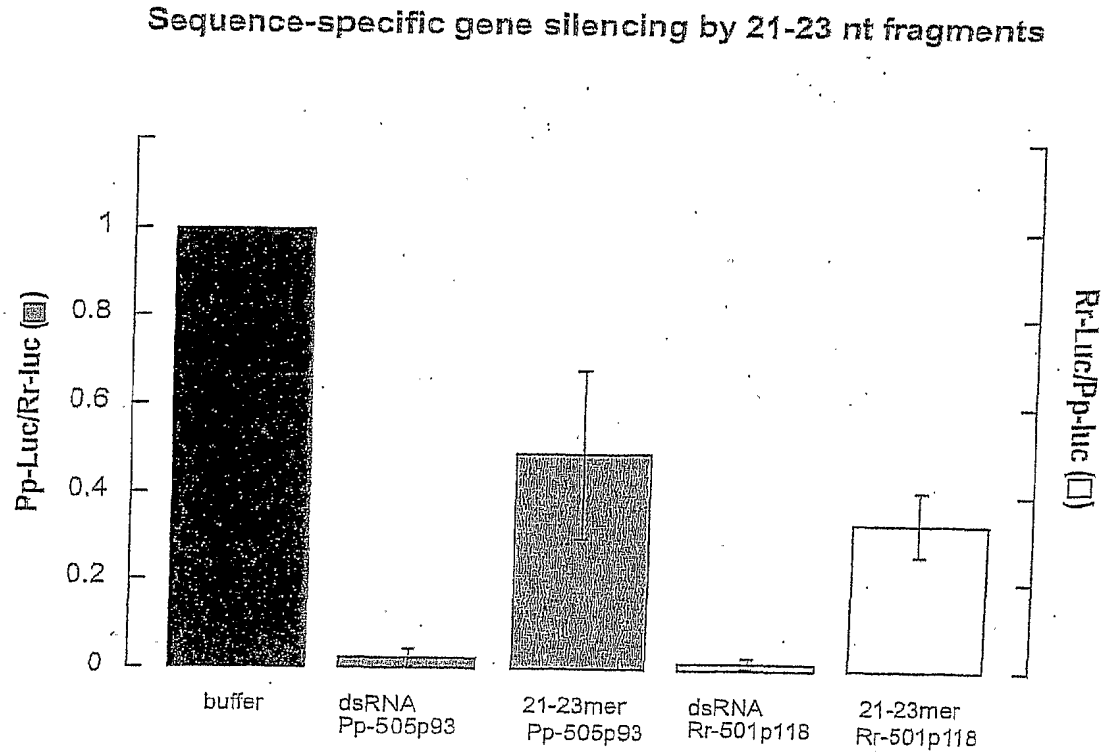


Figure 12

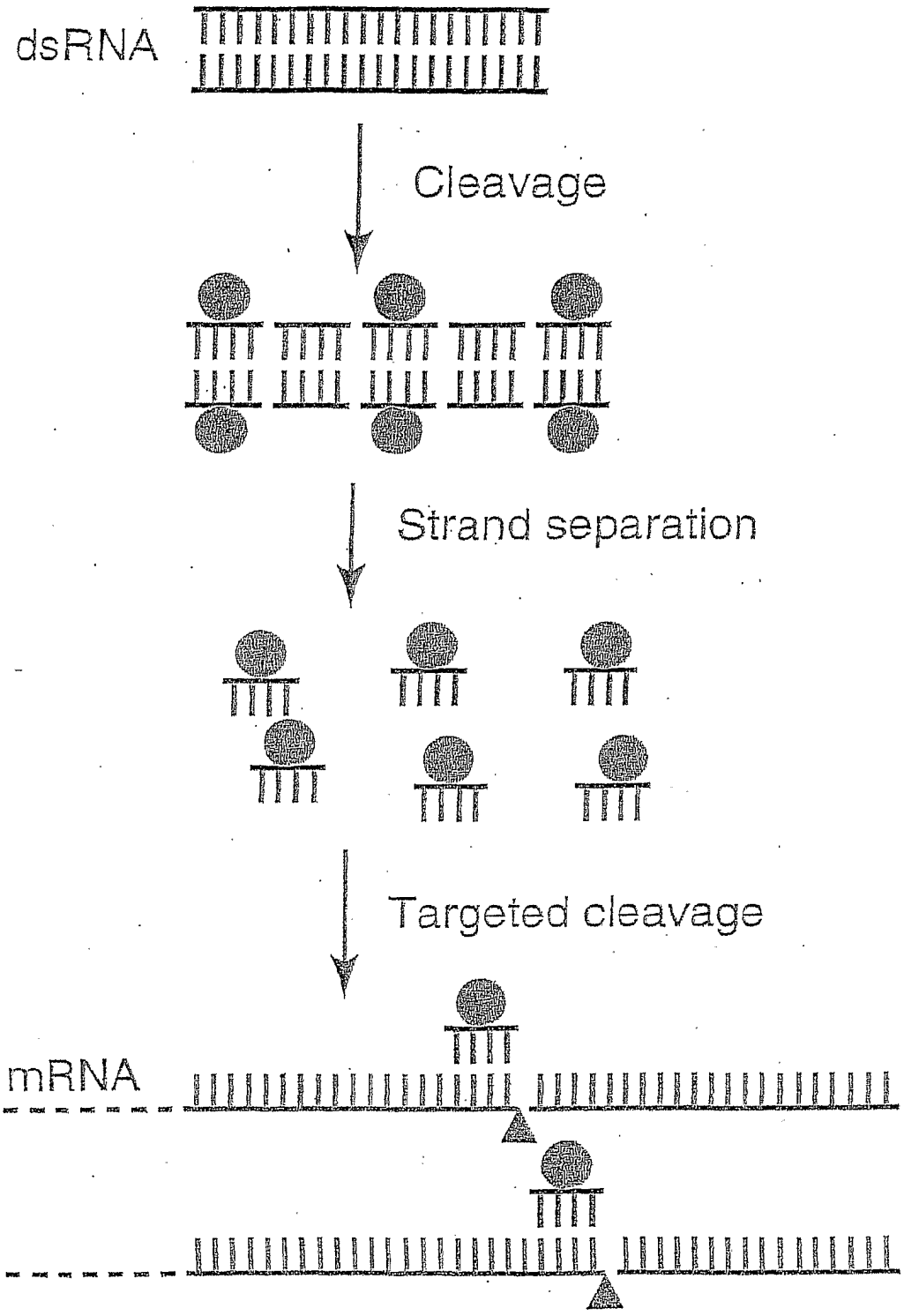
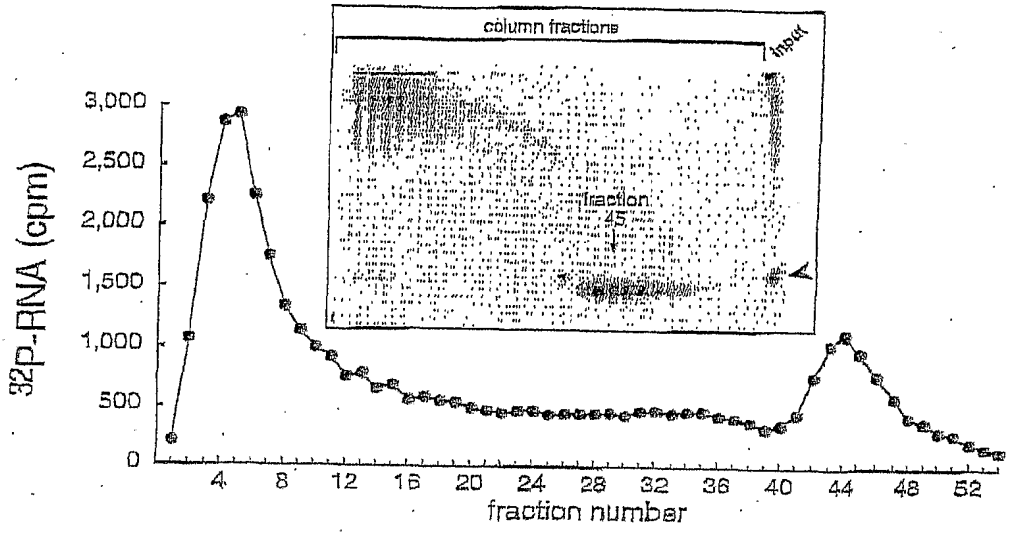


Figure 11

Figure 13A



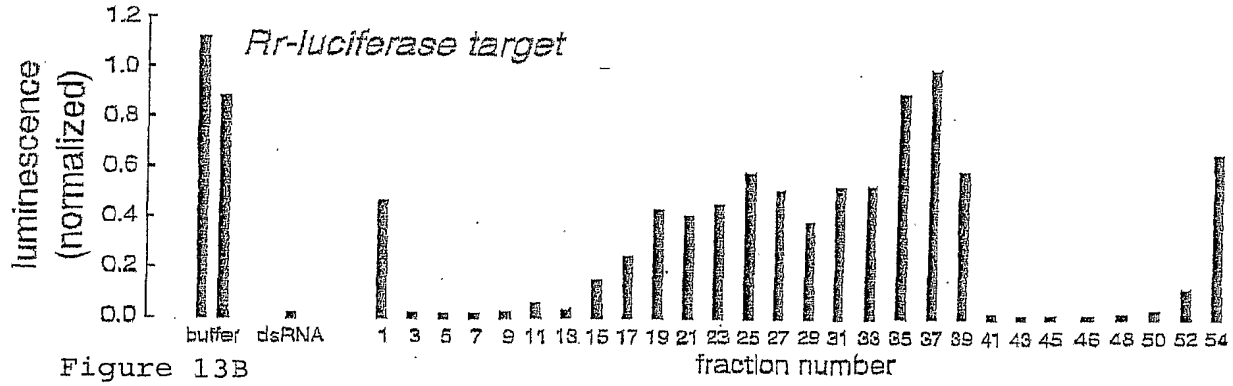


Figure 13B

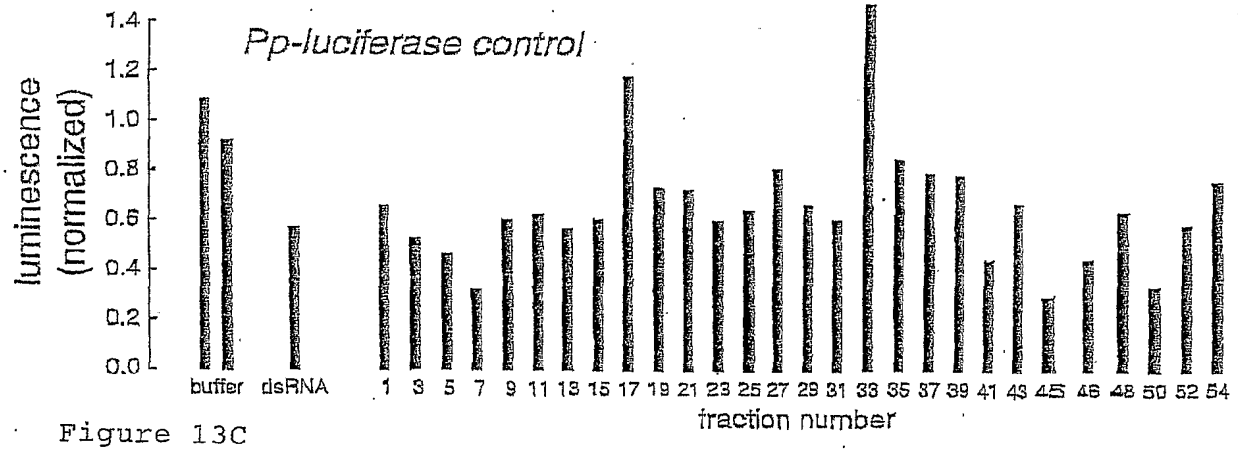


Figure 13C

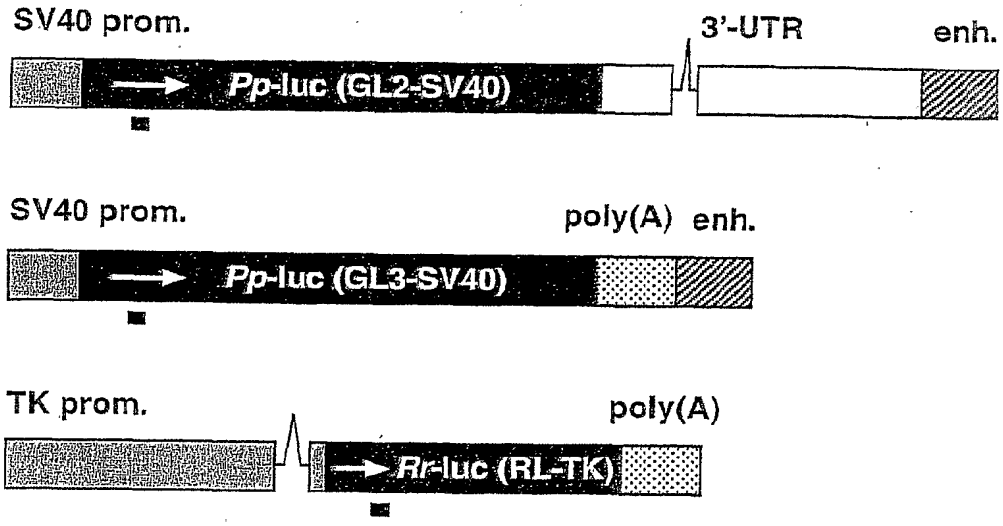


Figure 14A

siRNA duplex	
uGL2	5′ CGUACGCGGAAUACUUCGAUU UUGCAUGCGCCUUAUGAAGCU 5′
GL2	5′ CGUACGCGGAAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5′
GL3	5′ CUUACGCUGAGUACUUCGATT TTGAAUGCGACUCAUGAAGCU 5′
invGL2	5′ AGCUUCAUAAGGCGCAUGCTT TTUCGAAGUAUUCCGCGUACG 5′
RL	5′ AAACAUGCAGAAAAUGCUGTT TTUUUGUACGUCUUUUACGAC 5′

Figure 14B

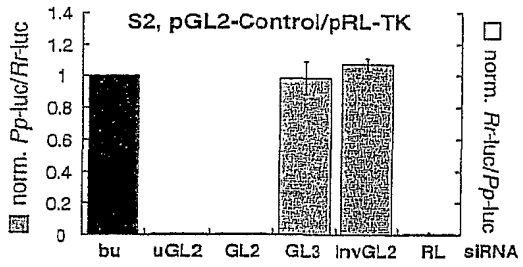


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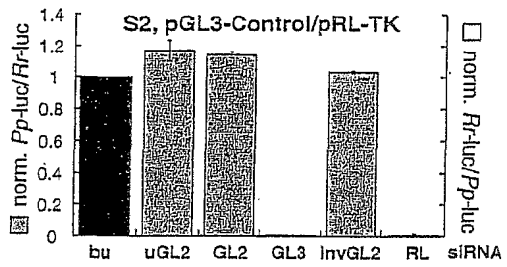


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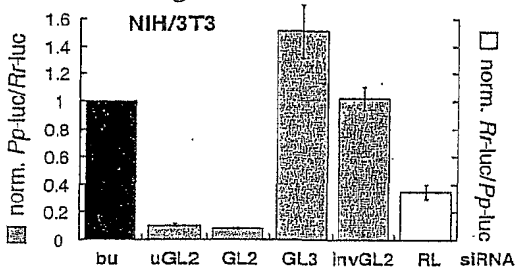


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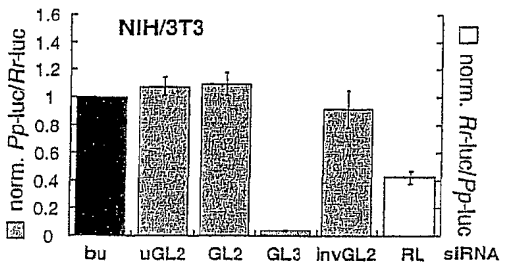


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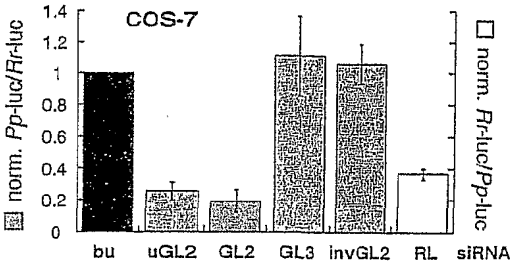


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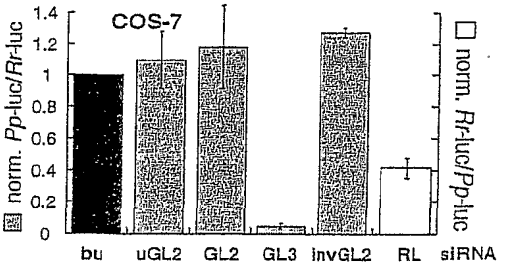


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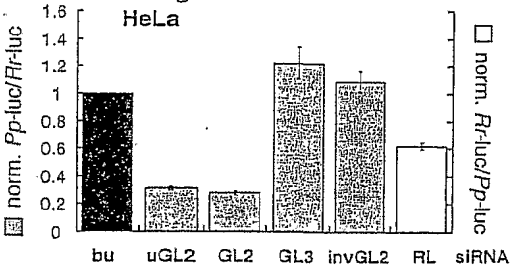


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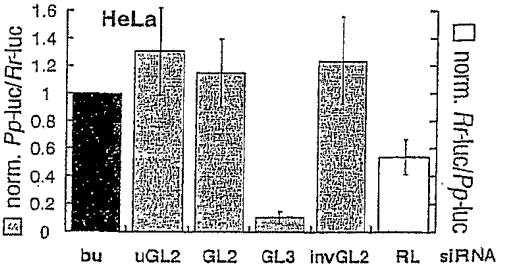


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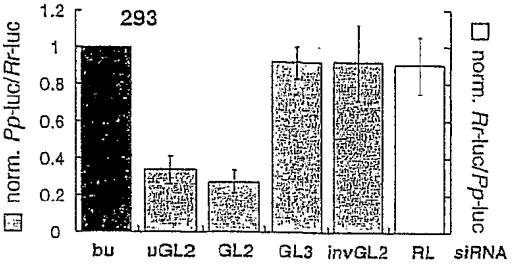


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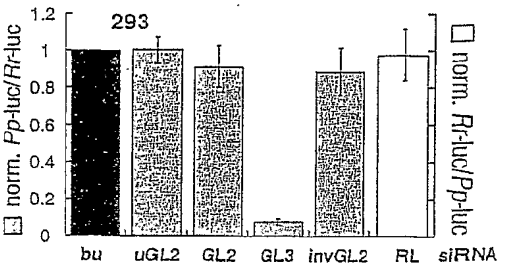


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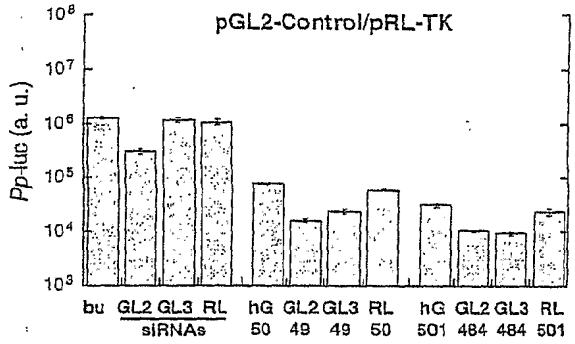


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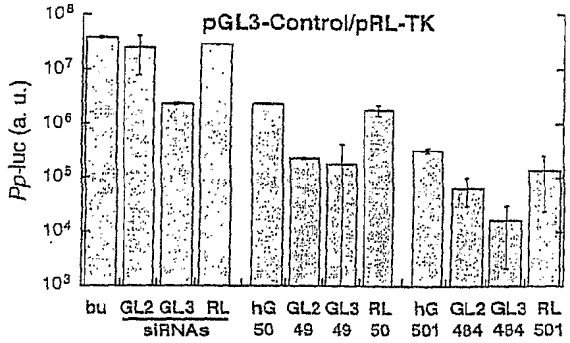


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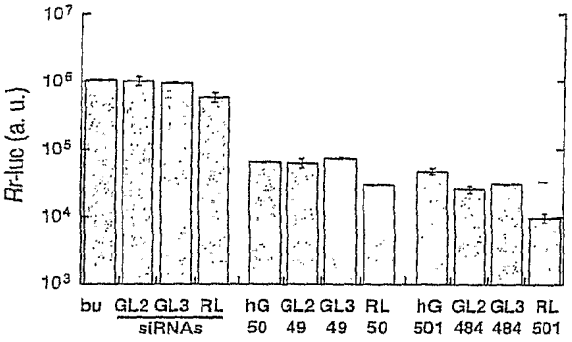


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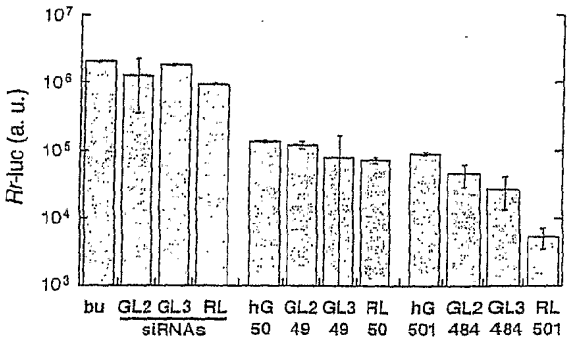


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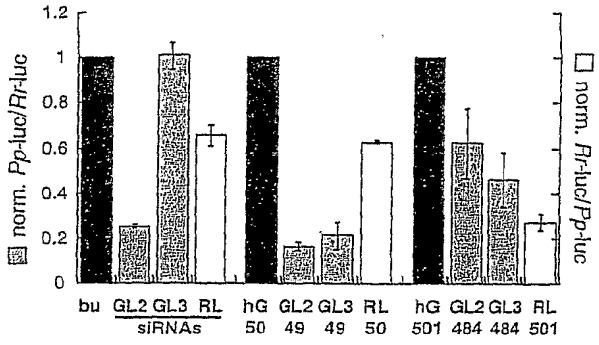


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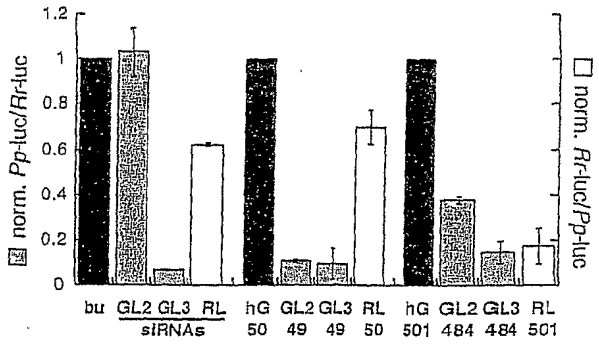


Figure 16F

EXHIBIT 6

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(12) **Patent Application Publication** (10) **Pub. No.: US 2002/0086356 A1**

Tuschl et al. (43) **Pub. Date: Jul. 4, 2002**

(54) **RNA SEQUENCE-SPECIFIC MEDIATORS OF RNA INTERFERENCE**

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(73) Assignee: **Whitehead Institute for Biomedical Research**, Cambridge, MA

(21) Appl. No.: **09/821,832**

(22) Filed: **Mar. 30, 2001**

Related U.S. Application Data

(63) Non-provisional of provisional application No. 60/265,232, filed on Jan. 31, 2001. Non-provisional of provisional application No. 60/193,594, filed on Mar. 30, 2000.

(30) **Foreign Application Priority Data**

Dec. 1, 2000 (EP) 00 126 325.0

Publication Classification

(51) **Int. Cl.**[7] **C12P 21/02**; C07H 21/04; C12N 9/22; C12P 19/34; C12N 5/06

(52) **U.S. Cl.** **435/69.1**; 435/348; 536/23.2; 435/91.2; 435/199

(57) **ABSTRACT**

The present invention relates to a Drosophila in vitro system which was used to demonstrate that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length. Furthermore, when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of long dsRNA. Thus, these 21-23 nt fragments are the sequence-specific mediators of RNA degradation. A molecular signal, which may be their specific length, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells, where the use of long dsRNAs to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. This specific targeting of a particular gene function is useful in functional genomic and therapeutic applications.

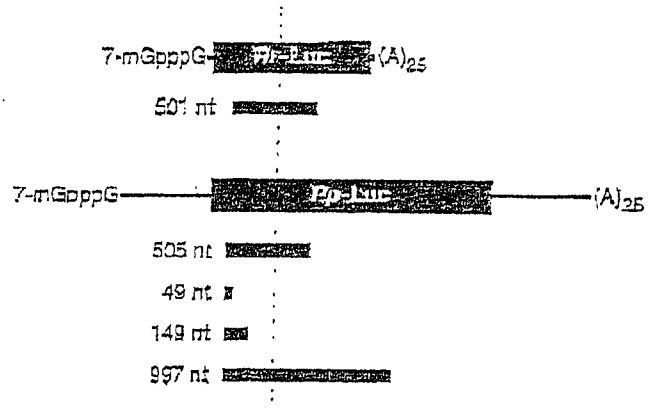


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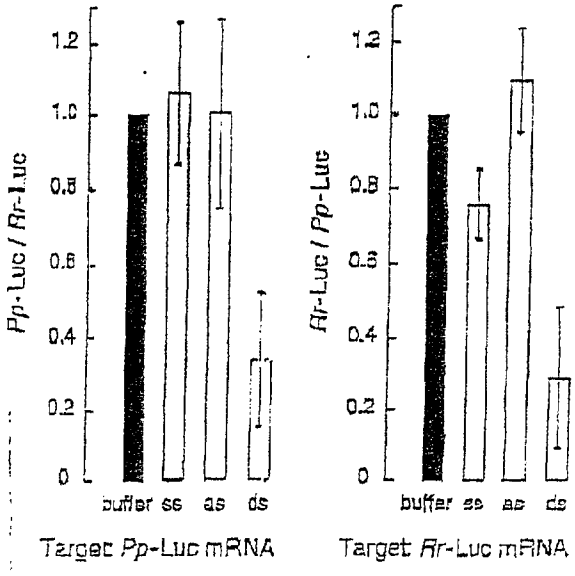


Figure 2A

Figure 2B

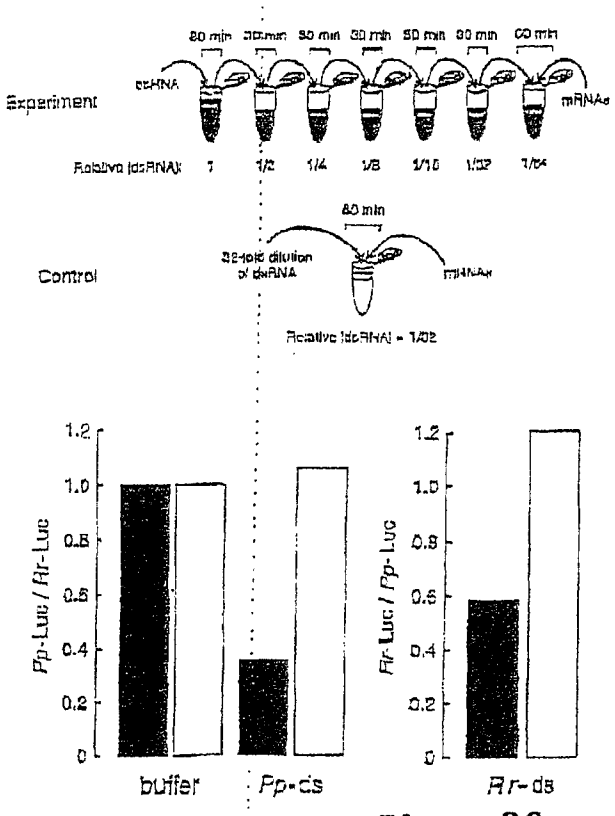


Figure 3A

Figure 3B

Figure 3C

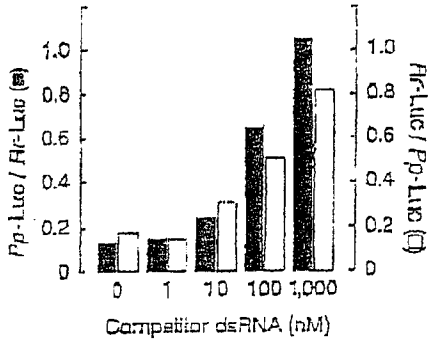


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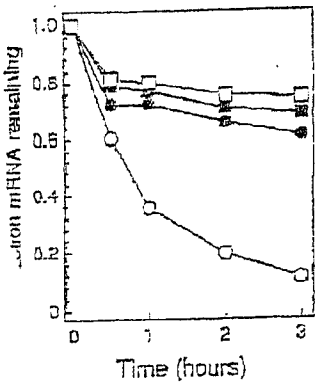


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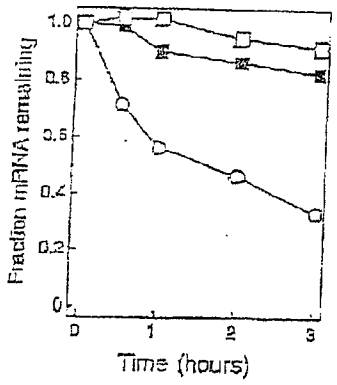


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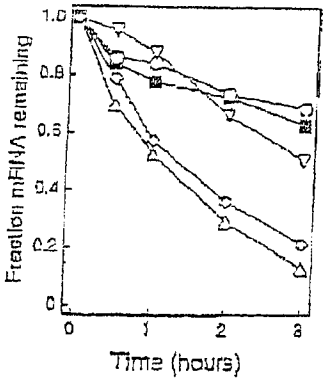


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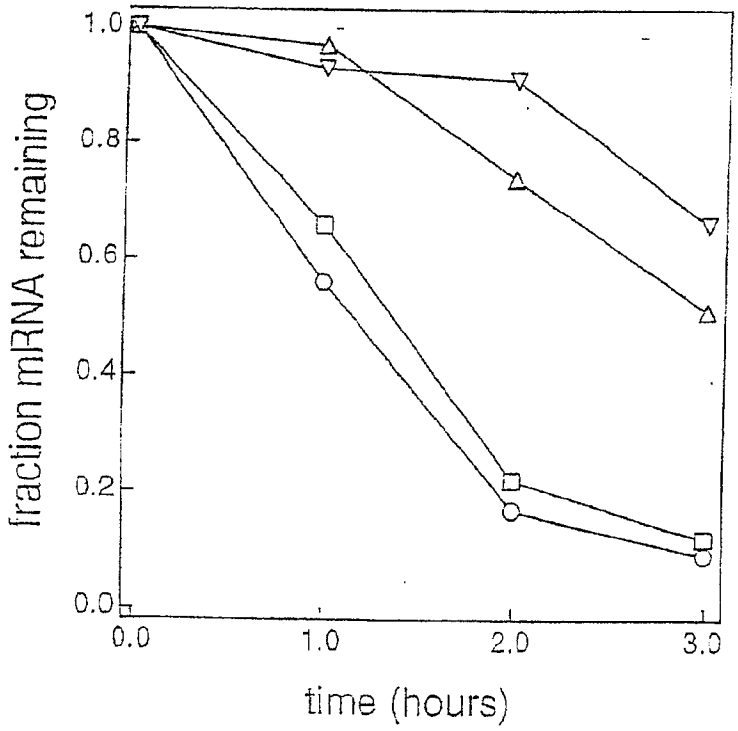


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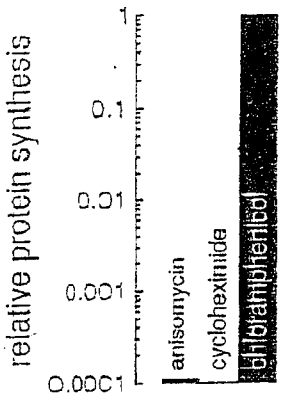


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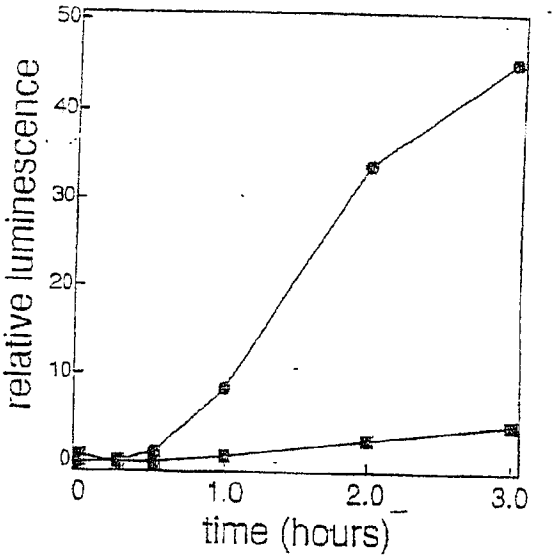


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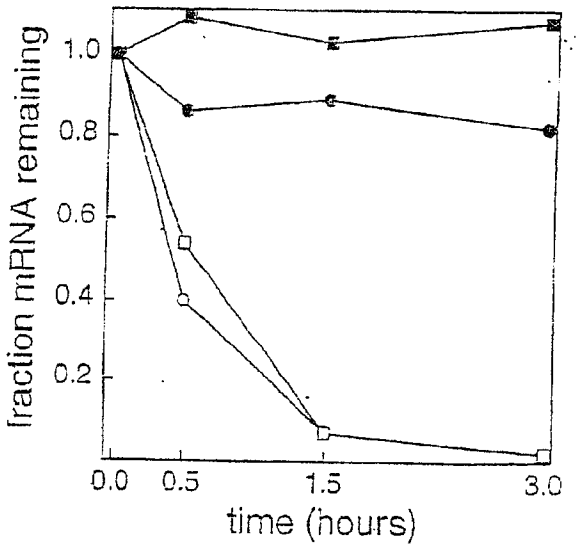


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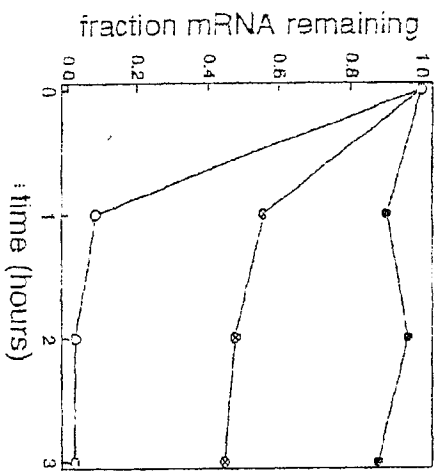


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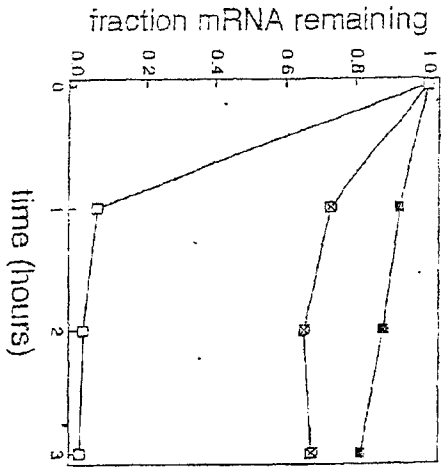


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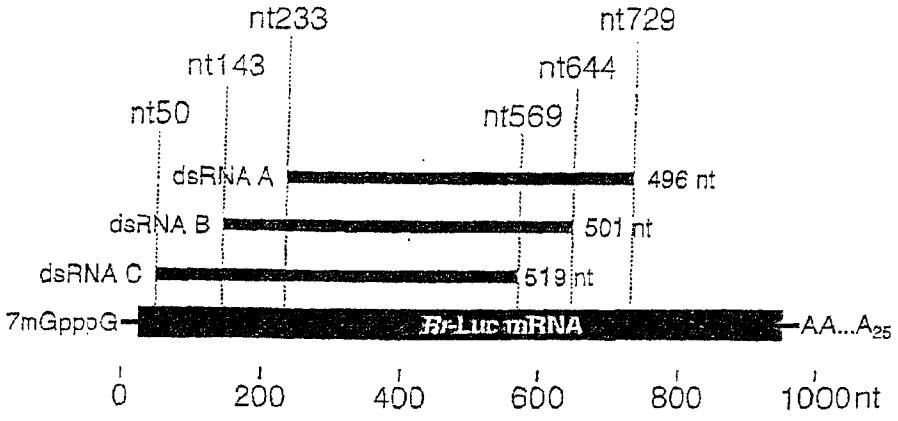
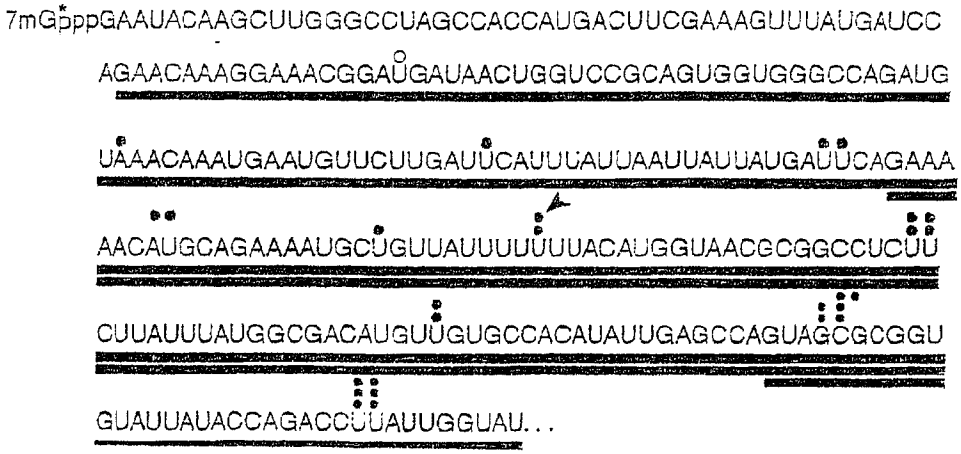


Figure 9

Figure 10



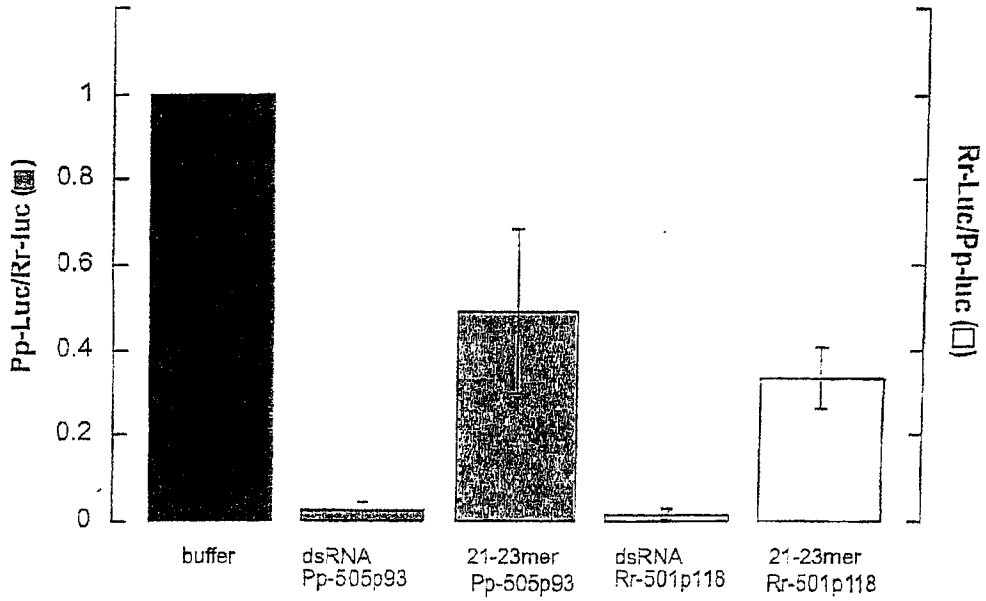


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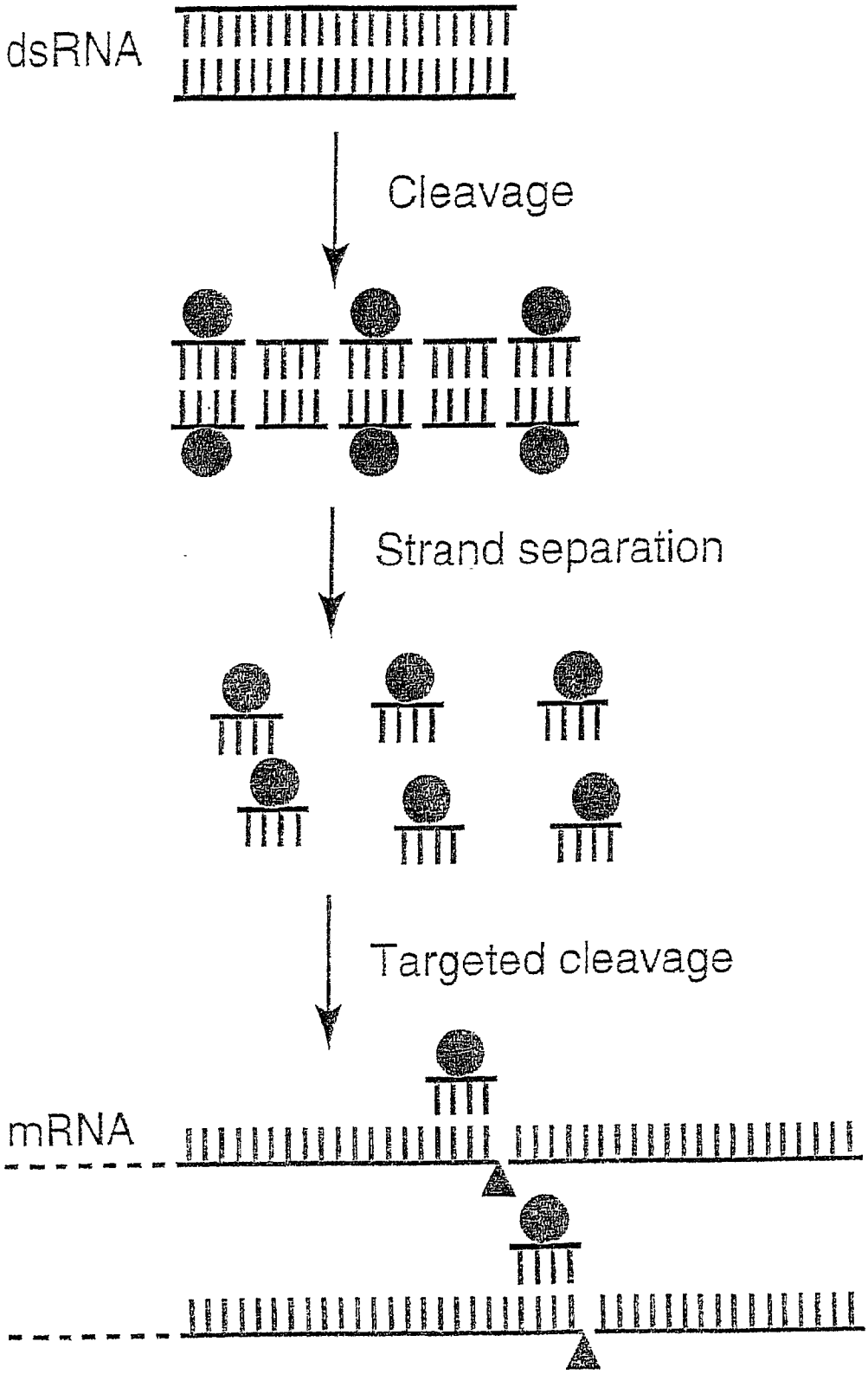
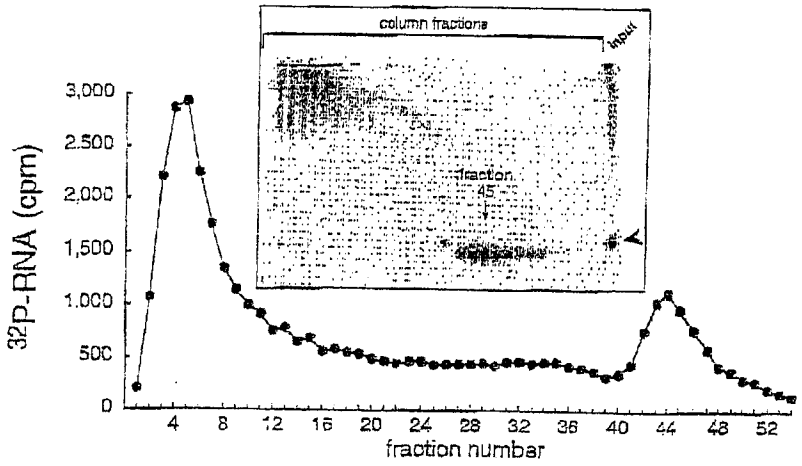


Figure 11

Figure 13A



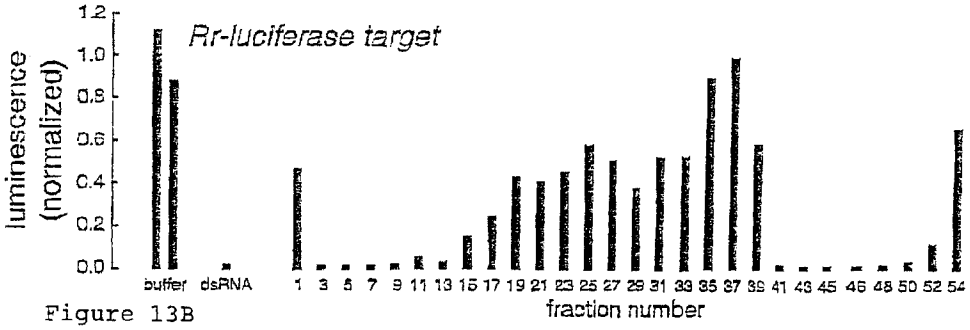


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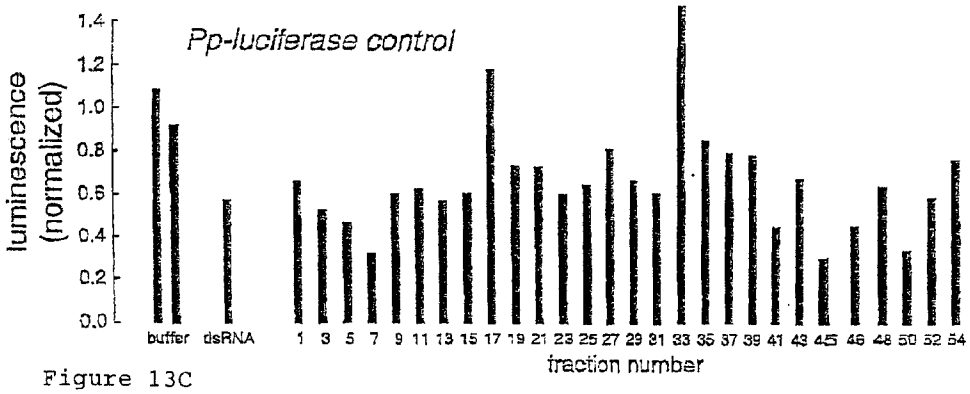


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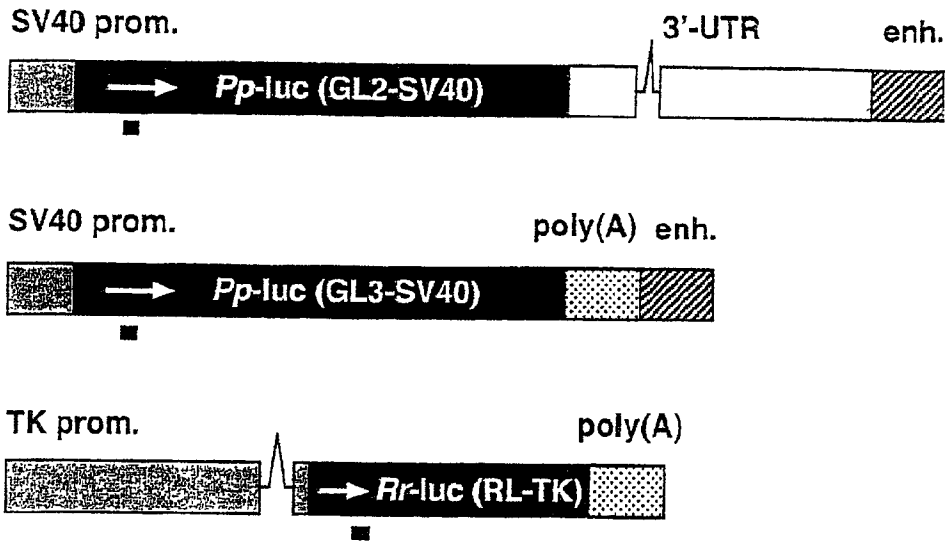


Figure 14A

siRNA duplex	
uGL2	5′ CGUACGCGGAAUACUUCGAUU UUGCAUGCGCCUUAUGAAGCU 5′
GL2	5′ CGUACGCGGAAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5′
GL3	5′ CUUACGCUGAGUACUUCGATT TTGAAUGCGACUCAUGAAGCU 5′
invGL2	5′ AGCUUCAUAAGGCGCAUGCTT TTUCGAAGUAUUCCGCGUACG 5′
RL	5′ AAACAUGCAGAAAAUGCUGTT TTUUUGUACGUCUUUUACGAC 5′

Figure 14B

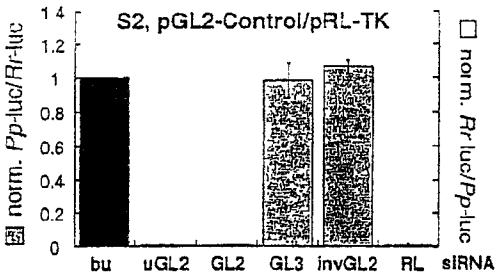


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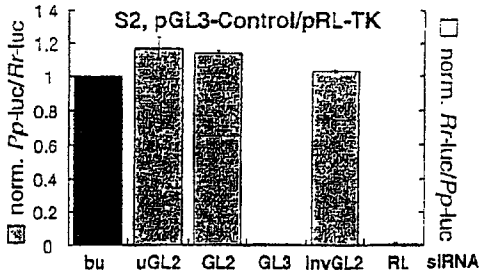


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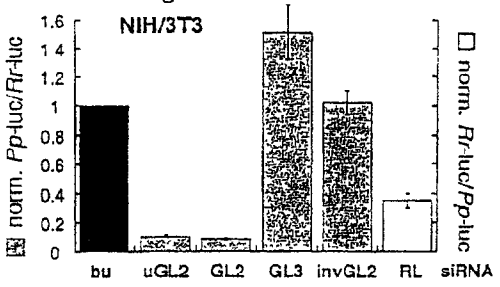


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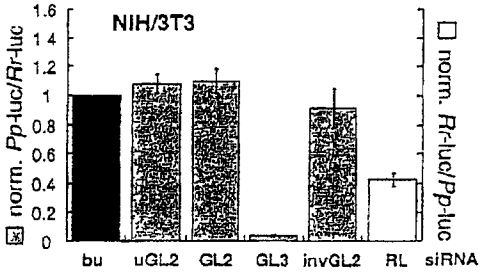


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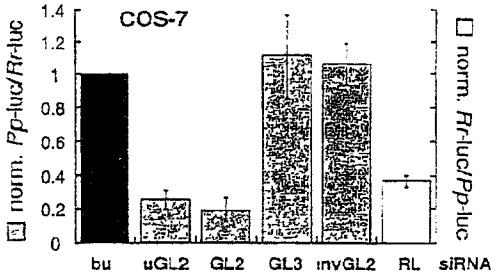


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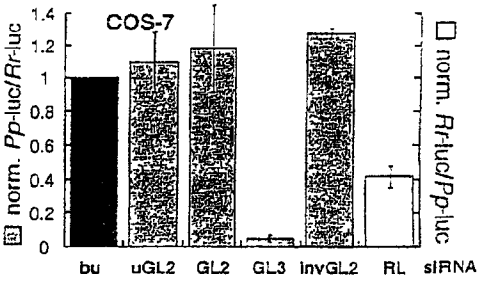


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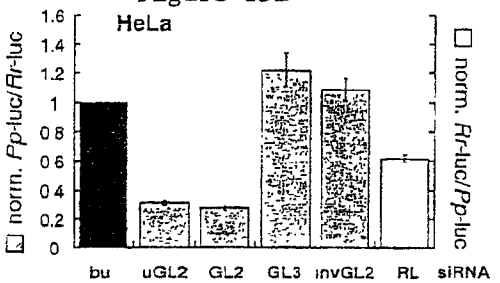


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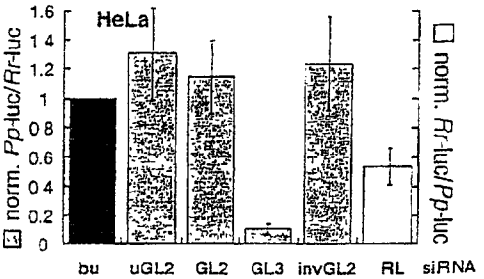


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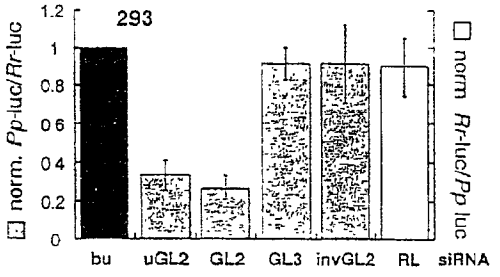


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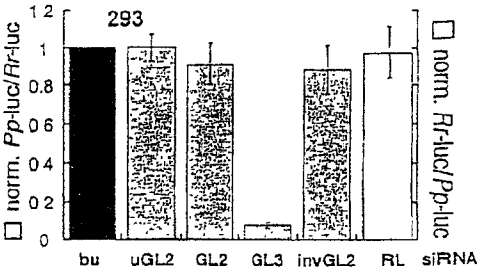


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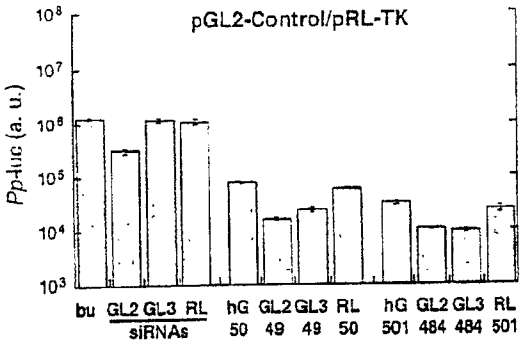


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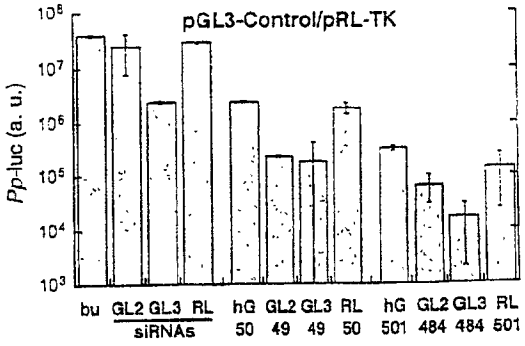


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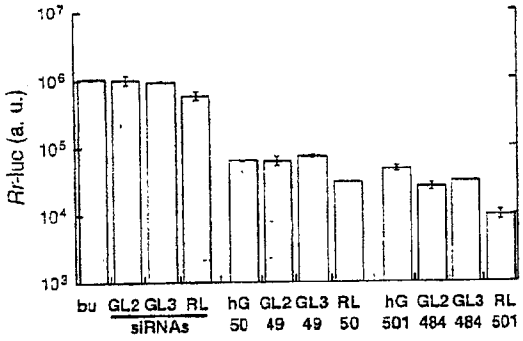


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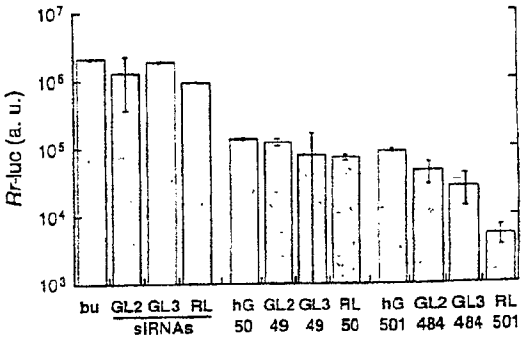


Figure 16D

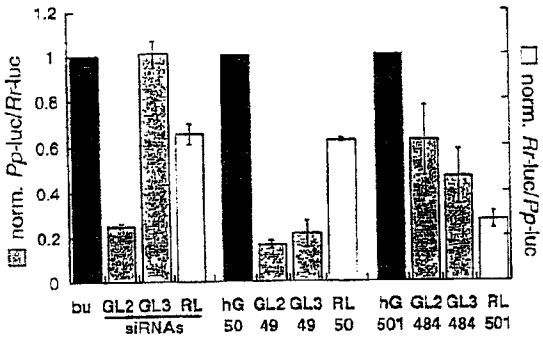


Figure 16E

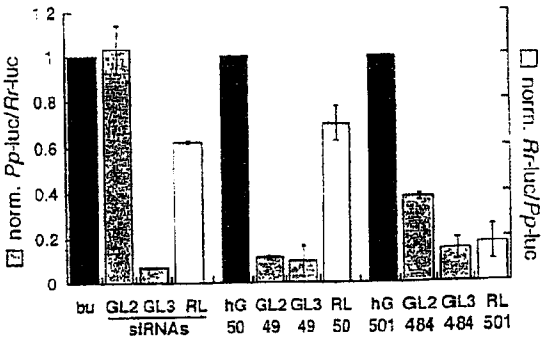


Figure 16F

RNA SEQUENCE-SPECIFIC MEDIATORS OF RNA INTERFERENCE

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/265,232, filed Jan. 31, 2001 and U.S. Provisional Application No. 60/193,594, filed Mar. 30, 2000, and claims priority under 35 U.S.C. §119 to European Application No. 00 126 325.0 filed Dec. 1, 2000. The entire teachings of the above applications are incorporated herein by reference.

GOVERNMENT SUPPORT

[0002] Work described herein was funded in part by grants from the National Institutes of Health through a United States Public Health Service MERIT award (Grant No. RO1-GM34277) from the National Institutes of Health. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (Fire et al. (1998) Nature 391, 806-811). dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. RNAi involves mRNA degradation, but many of the biochemical mechanisms underlying this interference are unknown. The recapitulation of the essential features of RNAi in vitro is needed for a biochemical analysis of the phenomenon.

SUMMARY OF THE INVENTION

[0004] Described herein is gene-specific, dsRNA-mediated interference in a cell-free system derived from syncytial blastoderm Drosophila embryos. The in vitro system complements genetic approaches to dissecting the molecular basis of RNAi. As described herein, the molecular mechanisms underlying RNAi were examined using the Drosophila in vitro system. Results showed that RNAi is ATP-dependent yet uncoupled from mRNA translation. That is, protein synthesis is not required for RNAi in vitro. In the RNAi reaction, both strands (sense and antisense) of the dsRNA are processed to small RNA fragments or segments of from about 21 to about 23 nucleotides (nt) in length (RNAs with mobility in sequencing gels that correspond to markers that are 21-23 nt in length, optionally referred to as 21-23 nt RNA). Processing of the dsRNA to the small RNA fragments does not require the targeted mRNA, which demonstrates that the small RNA species is generated by processing of the dsRNA and not as a product of dsRNA-targeted mRNA degradation. The mRNA is cleaved only within the region of identity with the dsRNA. Cleavage occurs at sites 21-23 nucleotides apart, the same interval observed for the dsRNA itself, suggesting that the 21-23 nucleotide fragments from the dsRNA are guiding mRNA cleavage. That purified 21-23 nt RNAs mediate RNAi confirms that these fragments are guiding mRNA cleavage.

[0005] Accordingly, the present invention relates to isolated RNA molecules (double-stranded; single-stranded) of from about 21 to about 23 nucleotides which mediate RNAi. That is, the isolated RNAs of the present invention mediate degradation of mRNA of a gene to which the mRNA corresponds (mediate degradation of mRNA that is the transcriptional product of the gene, which is also referred to as a target gene). For convenience, such mRNA is also referred to herein as mRNA to be degraded. As used herein, the terms RNA, RNA molecule(s), RNA segment(s) and RNA fragment(s) are used interchangeably to refer to RNA that mediates RNA interference. These terms include double-stranded RNA, single-stranded RNA, isolated RNA (partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA), as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the 21-23 nt RNA or internally (at one or more nucleotides of the RNA). Nucleotides in the RNA molecules of the present invention can also comprise non-standard nucleotides, including non-naturally occurring nucleotides or deoxyribonucleotides. Collectively, all such altered RNAs are referred to as analogs or analogs of naturally-occurring RNA. RNA of 21-23 nucleotides of the present invention need only be sufficiently similar to natural RNA that it has the ability to mediate (mediates) RNAi. As used herein the phrase "mediates RNAi" refers to (indicates) the ability to distinguish which RNAs are to be degraded by the RNAi machinery or process. RNA that mediates RNAi interacts with the RNAi machinery such that it directs the machinery to degrade particular mRNAs. In one embodiment, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that direct cleavage of specific mRNA to which their sequence corresponds. It is not necessary that there be perfect correspondence of the sequences, but the correspondence must be sufficient to enable the RNA to direct RNAi cleavage of the target mRNA. In a particular embodiment, the 21-23 nt RNA molecules of the present invention comprise a 3' hydroxyl group.

[0006] The present invention also relates to methods of producing RNA molecules of about 21 to about 23 nucleotides with the ability to mediate RNAi cleavage. In one embodiment, the Drosophila in vitro system is used. In this embodiment, dsRNA is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA molecules of about 21 to about 23 nucleotides. In another embodiment, the Drosophila in vitro system is used to obtain RNA sequences of about 21 to about 23 nucleotides which mediate RNA interference of the mRNA of a particular gene (e.g., oncogene, viral gene). In this embodiment, double-stranded RNA that corresponds to a sequence of the gene to be targeted is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides in length. As shown herein, 21-23 nt RNA mediates RNAi of the mRNA of the targeted gene (the gene whose mRNA is to be degraded). The method of obtaining 21-23 nt RNAs using the Drosophila in vitro system can further comprise isolating the RNA sequence from the combination.

[0007] The present invention also relates to 21-23 nt RNA produced by the methods of the present invention, as well as to 21-23 nt RNAs, produced by other methods, such as

chemical synthesis or recombinant DNA techniques, that have the same or substantially the same sequences as naturally-occurring RNAs that mediate RNAi, such as those produced by the methods of the present invention. All of these are referred to as 21-23 nt RNAs that mediate RNA interference. As used herein, the term isolated RNA includes RNA obtained by any means, including processing or cleavage of dsRNA as described herein; production by chemical synthetic methods; and production by recombinant DNA techniques. The invention further relates to uses of the 21-23 nt RNAs, such as for therapeutic or prophylactic treatment and compositions comprising 21-23 nt RNAs that mediate RNAi, such as pharmaceutical compositions comprising 21-23 nt RNAs and an appropriate carrier (e.g., a buffer or water).

[0008] The present invention also relates to a method of mediating RNA interference of mRNA of a gene in a cell or organism (e.g., mammal such as a mouse or a human). In one embodiment, RNA of about 21 to about 23 nt which targets the mRNA to be degraded is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of the mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism. The cell or organism can be one in which RNAi occurs as the cell or organism is obtained or a cell or organism can be one that has been modified so that RNAi occurs (e.g., by addition of components obtained from a cell or cell extract that mediate RNAi or activation of endogenous components). As used herein, the term "cell or organism in which RNAi occurs" includes both a cell or organism in which RNAi occurs as the cell or organism is obtained, or a cell or organism that has been modified so that RNAi occurs. In another embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNAs of about 21 to about 23 nucleotides. 21 to 23 nt RNA is then isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the gene in the cell or organism. As described for the previous embodiment, the cell or organism is one in which RNAi occurs naturally (in the cell or organism as obtained) or has been modified in such a manner that RNAi occurs. 21 to 23 nt RNAs can also be produced by other methods, such as chemical synthetic methods or recombinant DNA techniques.

[0009] The present invention also relates to biochemical components of a cell, such as a Drosophila cell, that process dsRNA to RNA of about 21 to about 23 nucleotides. In addition, biochemical components of a cell that are involved in targeting of mRNA by RNA of about 21 to about 23 nucleotides are the subject of the present invention. In both embodiments, the biochemical components can be obtained from a cell in which they occur or can be produced by other methods, such as chemical synthesis or recombinant DNA methods. As used herein, the term "isolated" includes materials (e.g., biochemical components, RNA) obtained from a source in which they occur and materials produced by methods such as chemical synthesis or recombinant nucleic acid (DNA, RNA) methods.

[0010] The present invention also relates to a method for knocking down (partially or completely) the targeted gene, thus providing an alternative to presently available methods of knocking down (or out) a gene or genes. This method of knocking down gene expression can be used therapeutically or for research (e.g., to generate models of disease states, to examine the function of a gene, to assess whether an agent acts on a gene, to validate targets for drug discovery). In those instances in which gene function is eliminated, the resulting cell or organism can also be referred to as a knockout. One embodiment of the method of producing knockdown cells and organisms comprises introducing into a cell or organism in which a gene (referred to as a targeted gene) is to be knocked down, RNA of about 21 to about 23 nt that targets the gene and maintaining the resulting cell or organism under conditions under which RNAi occurs, resulting in degradation of the mRNA of the targeted gene, thereby producing knockdown cells or organisms. Knockdown cells and organisms produced by the present method are also the subject of this invention.

[0011] The present invention also relates to a method of examining or assessing the function of a gene in a cell or organism. In one embodiment, RNA of about 21 to about 23 nt which targets mRNA of the gene for degradation is introduced into a cell or organism in which RNAi occurs. The cell or organism is referred to as a test cell or organism. The test cell or organism is maintained under conditions under which degradation of mRNA of the gene occurs. The phenotype of the test cell or organism is then observed and compared to that of an appropriate control cell or organism, such as a corresponding cell or organism that is treated in the same manner except that the targeted (specific) gene is not targeted. A 21 to 23 nt RNA that does not target the mRNA for degradation can be introduced into the control cell or organism in place of the RNA introduced into the test cell or organism, although it is not necessary to do so. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the degraded mRNA. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract that mediates RNAi, such as the soluble extract derived from Drosophila embryo described herein, under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into a cell or organism in which RNAi occurs (test cell or test organism). The test cell or test organism is maintained under conditions under which degradation of the mRNA occurs. The phenotype of the test cell or organism is then observed and compared to that of an appropriate control, such as a corresponding cell or organism that is treated in the same manner as the test cell or organism except that the targeted gene is not targeted. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the targeted gene. The information provided may be sufficient to identify (define) the function of the gene or may be used in conjunction with information obtained from other assays or analyses to do so.

[0012] Also the subject of the present invention is a method of validating whether an agent acts on a gene. In this method, RNA of from about 21 to about 23 nucleotides that targets the mRNA to be degraded is introduced into a cell or organism in which RNAi occurs. The cell or organism

(which contains the introduced RNA) is maintained under conditions under which degradation of mRNA occurs, and the agent is introduced into the cell or organism. Whether the agent has an effect on the cell or organism is determined; if the agent has no effect on the cell or organism, then the agent acts on the gene.

[0013] The present invention also relates to a method of validating whether a gene product is a target for drug discovery or development. RNA of from about 21 to about 23 nucleotides that targets the mRNA that corresponds to the gene for degradation is introduced into a cell or organism. The cell or organism is maintained under conditions in which degradation of the mRNA occurs, resulting in decreased expression of the gene. Whether decreased expression of the gene has an effect on the cell or organism is determined, wherein if decreased expression of the gene has an effect, then the gene product is a target for drug discovery or development.

[0014] The present invention also encompasses a method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNA of from about 21 to about 23 nucleotides which targets the mRNA of the protein (the mRNA that encodes the protein) for degradation. As a result, the protein is not produced or is not produced to the extent it would be in the absence of the treatment.

[0015] Also encompassed by the present invention is a gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

[0016] Also encompassed by the present invention is a method of identifying target sites within an mRNA that are particularly suitable for RNAi as well as a method of assessing the ability of 21-23 nt RNAs to mediate RNAi.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

[0018] **FIG. 1** is a schematic representation of reporter mRNAs and dsRNAs Rr-Luc and Pp-Luc. Lengths and positions of the ssRNA, asRNA, and dsRNAs are shown as black bars relative to the Rr-Luc and Pp-Luc reporter mRNA sequences. Black rectangles indicate the two unrelated luciferase coding sequences, lines correspond to the 5' and 3' untranslated regions of the mRNAs.

[0019] **FIG. 2A** is a graph of the ratio of luciferase activities after targeting 50 pM Pp-Luc mRNA with 10 nM ssRNA, asRNA, or dsRNA from the 505 bp segment of the Pp-Luc gene showing gene-specific interference by dsRNA in vitro. The data are the average values of seven trials ±standard deviation. Four independently prepared lysates were used. Luciferase activity was normalized to the buffer control; a ratio equal to one indicates no gene-specific interference.

[0020] **FIG. 2B** is a graph of the ratio of luciferase activities after targeting 50 pM Rr-Luc mRNA with 10 nM ssRNA, asRNA, or dsRNA from the 501 bp segment of the Rr-Luc gene showing gene-specific interference by dsRNA in vitro. The data are the average values of six trials ±standard deviation. A Rr-Luc/Pp-Luc ratio equal to one indicates no gene-specific interference.

[0021] **FIG. 3A** is a schematic representation of the experimental strategy used to show that incubation in the Drosophila embryo lysate potentiates dsRNA for gene-specific interference. The same dsRNAs used in **FIG. 2** (or buffer) was serially preincubated using two-fold dilutions in six successive reactions with Drosophila embryo lysate, then tested for its capacity to block mRNA expression. As a control, the same amount of dsRNA (10 nM) or buffer was diluted directly in buffer and incubated with Pp-Luc and Rr-Luc mRNAs and lysate.

[0022] **FIG. 3B** is a graph of potentiation when targeting Pp-Luc mRNA. Black columns indicate the dsRNA or the buffer was serially preincubated; white columns correspond to a direct 32-fold dilution of the dsRNA. Values were normalized to those of the buffer controls.

[0023] **FIG. 3C** is a graph of potentiation when targeting Rr-Luc mRNA. The corresponding buffer control is shown in **FIG. 3B**.

[0024] **FIG. 4** is a graph showing effect of competitor dsRNA on gene-specific interference. Increasing concentrations of nanos dsRNA (508 bp) were added to reactions containing 5 nM dsRNA (the same dsRNAs used in **FIGS. 2A and 2B**) targeting Pp-Luc mRNA (black columns, left axis) or Rr-Luc mRNA (white columns, right axis). Each reaction contained both a target mRNA (Pp-Luc for the black columns, Rr-Luc for the white) and an unrelated control mRNA (Rr-Luc for the black columns, Pp-Luc for the white). Values were normalized to the buffer control (not shown). The reactions were incubated under standard conditions (see Methods).

[0025] **FIG. 5A** is a graph showing the effect of dsRNA on mRNA stability. Circles, Pp-Luc mRNA; squares, Rr-Luc mRNA; filled symbols, buffer incubation; open symbols, incubation with Pp-dsRNA.

[0026] **FIG. 5B** is a graph showing the stability of Rr-Luc mRNA incubated with Rr-dsRNA or Pp-dsRNA. Filled squares, buffer; open squares, Pp-dsRNA (10 nM); open circles, Rr-dsRNA (10 nM).

[0027] **FIG. 5C** is a graph showing the dependence on dsRNA length. The stability of the Pp-Luc mRNA was assessed after incubation in lysate in the presence of buffer or dsRNAs of different lengths. Filled squares, buffer; open circles, 49 bp dsRNA (10 nM); open inverted triangles, 149 bp dsRNA (10 nM); open triangles, 505 bp dsRNA (10 nM); open diamonds, 997 bp dsRNA (10 nM). Reactions were incubated under standard conditions (see Methods).

[0028] **FIG. 6** is a graph showing that RNAi Requires ATP. Creatine kinase (CK) uses creatine phosphate (CP) to regenerate ATP. Circles, +ATP, +CP, +CK; squares, −ATP, +CP, +CK; triangles, −ATP, −CP, +CK; inverted triangles, −ATP, +CP, −CK.

[0029] **FIG. 7A** is a graph of protein synthesis, as reflected by luciferase activity produced after incubation of Rr-luc mRNA in the in vitro RNAi reaction for 1 hour, in the presence of the protein synthesis inhibitors anisomycin, cycloheximide, or chloramphenicol, relative to a reaction without any inhibitor showing that RNAi does not require mRNA translation.

[0030] **FIG. 7B** is a graph showing translation of 7-methyl-guanosine- and adenosine-capped Pp-luc mRNAs (circles and squares, respectively) in the RNAi reaction in the absence of dsRNA, as measured by luciferase activity produced in a one-hour incubation.

[0031] **FIG. 7C** is a graph showing incubation in an RNAi reaction of uniformly ^{32}P-radiolabeled 7-methyl-guanosine-capped Pp-luc mRNA (circles) and adenosine-capped Pp-luc mRNA (squares), in the presence (open symbols) and absence (filled symbols) of 505 bp Pp-luc dsRNA.

[0032] **FIG. 8A** is a graph of the of the denaturing agarose-gel analysis of Pp-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt Pp-asRNA, or 505 bp Pp-dsRNA for the times indicated showing that asRNA causes a small amount of RNAi in vitro.

[0033] **FIG. 8B** is a graph of the of the denaturing agarose-gel analysis of Rr-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt Pp-asRNA, or 505 bp Pp-dsRNA for the times indicated showing that asRNA causes a small amount of RNAi in vitro.

[0034] **FIG. 9** is a schematic of the positions of the three dsRNAs, 'A,''B,' and 'C,' relative to the Rr-luc mRNA.

[0035] **FIG. 10** indicates the cleavage sites mapped onto the first 267 nt of the Rr-luc mRNA (SEQ ID NO: 1). The blue bar below the sequence indicates the position of dsRNA 'C,' and blue circles indicate the position of cleavage sites caused by this dsRNA. The green bar denotes the position of dsRNA 'B,' and green circles, the cleavage sites. The magenta bar indicates the position of dsRNA 'A,' and magenta circles, the cleavages. An exceptional cleavage within a run of 7 uracils is marked with a red arrowhead.

[0036] **FIG. 11** is a proposed model for RNAi. RNAi is envisioned to begin with cleavage of the dsRNA to 21-23 nt products by a dsRNA-specific nuclease, perhaps in a multiprotein complex. These short dsRNAs might then be dissociated by an ATP-dependent helicase, possibly a component of the initial complex, to 21-23 nt asRNAs that could then target the mRNA for cleavage. The short asRNAs are imagined to remain associated with the RNAi-specific proteins (circles) that were originally bound by the full-length dsRNA, thus explaining the inefficiency of asRNA to trigger RNAi in vivo and in vitro. Finally, a nuclease (triangles) would cleave the mRNA.

[0037] **FIG. 12** is a bar graph showing sequence-specific gene silencing by 21-23 nt fragments. Ratio of luciferase activity after targeting of Pp-Luc and Rr-Luc mRNA by 5 nM Pp-Luc or Rr-Luc dsRNA (500 bp) or 21-23 nt fragments isolated from a previous incubation of the respective dsRNA in Drosophila lysate. The amount of isolated 21-23 mers present in the incubation reaction correspond to approximately the same amount of 21-23 mers generated during an incubation reaction with 5 nM 500 bp dsRNA. The data are average values of 3 trials and the standard deviation is given by error bars. Luciferase activity was normalized to the buffer control.

[0038] **FIG. 13A** illustrates the purification of RNA fragments on a Superdex HR 200 10/30 gel filtration column (Pharmacia) using the method described in Example 4. dsRNA was 32P-labeled, and the radioactivity recovered in each column fraction is graphed. The fractions were also analyzed by denaturing gel electrophoresis (inset).

[0039] **FIG. 13B** demonstrates the ability of the Rr-luciferase RNA, after incubation in the Drosophila lysate and fractionation as in **FIG. 13A**, to mediate sequence-specific interference with the expression of a Rr-luciferase target mRNA. One microliter of each resuspended fraction was tested in a 10 microliter in vitro RNAi reaction (see Example 1). This procedure yields a concentration of RNA in the standard in vitro RNAi reaction that is approximately equal to the concentration of that RNA species in the original reaction prior to loading on the column. Relative luminescence per second has been normalized to the average value of the two buffer controls.

[0040] **FIG. 13C** is the specificity control for **FIG. 13B**. It demonstrates that the fractionated RNA of **FIG. 13B** does not efficiently mediate sequence-specific interference with the expression of a Pp-luciferase mRNA. Assays are as in **FIG. 13B**.

[0041] **FIGS. 14A and 14B** are schematic representations of reporter constructs and siRNA duplexes.

[0042] **FIG. 14A** illustrates the firefly (Pp-luc) and sea pansy (Rr-luc) luciferase reporter gene regions from plasmids pGL2-Control, pGL3-Control, and pRL-TK (Promega). SV40 regulatory elements, the HSV thymidine kinase promoter, and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase expression from pGL2 is approximately 10-fold lower than from pGL3 in transfected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes.

[0043] **FIG. 14B** shows the sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2 (SEQ ID Nos: 10 and 11), GL3 (SEQ ID Nos: 12 and 13), and RL (SEQ ID Nos: 14 and 15) luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only 3 single nucleotide substitutions (boxed in gray). As unspecific control, a duplex with the inverted GL2 sequence, invGL2 (SEQ ID Nos: 16 and 17), was synthesized. The 2 nt 3' overhang of 2'-deoxythymidine is indicated as TT; uGL2 (SEQ ID Nos: 18 and 19) is similar to GL2 siRNA but contains ribo-uridine 3' overhangs.

[0044] FIGS. **15A-15J** are graphs showing RNA interference by siRNA duplexes. Ratios of target to control luciferase were normalized to a buffer control (bu, black bars); gray bars indicate ratios of *Photinus pyralis* (Pp-luc) GL2 or GL3 luciferase to *Renilla reniformis* (Rr-luc) RL luciferase (left axis), white bars indicate RL to GL2 or GL3 ratios (right axis).

[0045] **FIGS. 15A, 15C, 15E, 15G,** and **15I** show results of experiments performed with the combination of pGL2-Control and pRL-TK reporter plasmids,

[0046] **FIGS. 15B, 15D, 15F, 15H,** and **15J** with pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of Pp-luc/Rr-luc for the buffer control (bu) varied between 0.5 and 10 for pGL2/pRL, and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and

between the various cell lines tested. The plotted data were averaged from three independent experiments ±S.D.

[0047] FIGS. 16A-16F are graphs showing the effects of 21 nt siRNAs, 50 bp, and 500 bp dsRNAs on luciferase expression in HeLa cells. The exact length of the long dsRNAs is indicated below the bars.

[0048] FIGS. 16A, 16C, and 16E describe experiments performed with pGL2-Control and pRL-TK reporter plasmids,

[0049] FIGS. 16B, 16D, and 16F with pGL3-Control and pRL-TK reporter plasmids. The data were averaged from two independent experiments ±S.D.

[0050] FIGS. 16A, 16B, Absolute Pp-luc expression, plotted in arbitrary luminescence units.

[0051] FIG. 16C, 16D, Rr-luc expression, plotted in arbitrary luminescence units.

[0052] FIGS. 16E, 16F, Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (bu, black bars); the luminescence ratios for 50 or 500 bp dsRNAs were normalized to the respective ratios observed for 50 and 500 bp dsRNA from humanized GFP (hG, black bars). It should be noted, that the overall differences in sequence between the 49 and 484 bp dsRNAs targeting GL2 and GL3 are not sufficient to confer specificity between GL2 and GL3 targets (43 nt uninterrupted identity in 49 bp segment, 239 nt longest uninterrupted identity in 484 bp segment) (Parrish, S., et al., *Mol. Cell,* 6:1077-1087 (2000)).

DETAILED DESCRIPTION OF THE INVENTION

[0053] Double-stranded (dsRNA) directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). The process is known to occur in a wide variety of organisms, including embryos of mammals and other vertebrates. Using the Drosophila in vitro system described herein, it has been demonstrated that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length, and furthermore, that when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of longer dsRNA. Thus, these 21-23 nt fragments are sequence-specific mediators of RNA degradation. A molecular signal, which may be the specific length of the fragments, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or recombinantly produced or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells. Use of long dsRNAs in mammalian cells to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. Specific targeting of a particular gene function, which is possible with 21-23 nt fragments of the present invention, is useful in functional genomic and therapeutic applications.

[0054] In particular, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that mediate RNAi. In one embodiment, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that direct cleavage of specific mRNA to which they correspond. The 21-23 nt RNA molecules of the present invention can also comprise a 3' hydroxyl group. The 21-23 nt RNA molecules can be single-stranded or double stranded (as two 21-23 nt RNAs); such molecules can be blunt ended or comprise overhanging ends (e.g., 5', 3'). In specific embodiments, the RNA molecule is double stranded and either blunt ended or comprises overhanging ends (as two 21-23 nt RNAs).

[0055] In one embodiment, at least one strand of the RNA molecule has a 3' overhang from about 1 to about 6 nucleotides (e.g., pyrimidine nucleotides, purine nucleotides) in length. In other embodiments, the 3' overhang is from about 1 to about 5 nucleotides, from about 1 to about 3 nucleotides and from about 2 to about 4 nucleotides in length. In one embodiment the RNA molecule is double stranded, one strand has a 3' overhang and the other strand can be blunt-ended or have an overhang. In the embodiment in which the RNA molecule is double stranded and both strands comprise an overhang, the length of the overhangs may be the same or different for each strand. In a particular embodiment, the RNA of the present invention comprises 21 nucleotide strands which are paired and which have overhangs of from about 1 to about 3, particularly about 2, nucleotides on both 3' ends of the RNA. In order to further enhance the stability of the RNA of the present invention, the 3' overhangs can be stabilized against degradation. In one embodiment, the RNA is stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine 2 nucleotide 3' overhangs by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNAi. The absence of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium.

[0056] The 21-23 nt RNA molecules of the present invention can be obtained using a number of techniques known to those of skill in the art. For example, the RNA can be chemically synthesized or recombinantly produced using methods known in the art. The 21-23 nt RNAs can also be obtained using the Drosophila in vitro system described herein. Use of the Drosophila in vitro system entails combining dsRNA with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA of about 21 to about 23 nucleotides. The Drosophila in vitro system can also be used to obtain RNA of about 21 to about 23 nucleotides in length which mediates RNA interference of the mRNA of a particular gene (e.g., oncogene, viral gene). In this embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to the RNA of about 21 to about 23 nucleotides. As shown herein, 21-23 nt RNA mediates RNAi of the mRNA to be degraded. The present invention also relates to the 21-23 nt RNA molecules produced by the methods described herein.

[0057] In one embodiment, the methods described herein are used to identify or obtain 21-23 nt RNA molecules that are useful as sequence-specific mediators of RNA degrada-

tion and, thus, for inhibiting mRNAs, such as human mRNAs, that encode products associated with or causative of a disease or an undesirable condition. For example, production of an oncoprotein or viral protein can be inhibited in humans in order to prevent the disease or condition from occurring, limit the extent to which it occurs or reverse it. If the sequence of the gene to be targeted in humans is known, 21-23 nt RNAs can be produced and tested for their ability to mediate RNAi in a cell, such as a human or other primate cell. Those 21-23 nt human RNA molecules shown to mediate RNAi can be tested, if desired, in an appropriate animal model to further assess their in vivo effectiveness. Additional copies of 21-23 nt RNAs shown to mediate RNAi can be produced by the methods described herein.

[0058] The method of obtaining the 21-23 nt RNA sequence using the Drosophila in vitro system can further comprise isolating the RNA sequence from the combination. The 21-23 nt RNA molecules can be isolated using a number of techniques known to those of skill in the art. For example, gel electrophoresis can be used to separate 21-23 nt RNAs from the combination, gel slices comprising the RNA sequences removed and RNAs eluted from the gel slices. Alternatively, non-denaturing methods, such as non-denaturing column chromatography, can be used to isolate the RNA produced. In addition, chromatography (e.g., size exclusion chromatography), glycerol gradient centrifugation, affinity purification with antibody can be used to isolate 21-23 nt RNAs. The RNA-protein complex isolated from the Drosophila in vitro system can also be used directly in the methods described herein (e.g., method of mediating RNAi of mRNA of a gene). Soluble extracts derived from Drosophila embryo that mediate or RNAi are encompassed by the invention. The soluble Drosophila extract can be obtained in a variety of ways. For example, the soluble extract can be obtained from syncytial blastoderm Drosophila embryos as described in Examples 1, 2, and 3. Soluble extracts can be derived from other cells in which RNAi occurs. Alternatively, soluble extracts can be obtained from a cell that does not carry out RNAi. In this instance, the factors needed to mediate RNAi can be introduced into such a cell and the soluble extract is then obtained. The components of the extract can also be chemically synthesized and/or combined using methods known in the art.

[0059] Any dsRNA can be used in the methods of the present invention, provided that it has sufficient homology to the targeted gene to mediate RNAi. The sequence of the dsRNA for use in the methods of the present invention need not be known. Alternatively, the dsRNA for use in the present invention can correspond to a known sequence, such as that of an entire gene (one or more) or portion thereof. There is no upper limit on the length of the dsRNA that can be used. For example, the dsRNA can range from about 21 base pairs (bp) of the gene to the full length of the gene or more. In one embodiment, the dsRNA used in the methods of the present invention is about 1000 bp in length. In another embodiment, the dsRNA is about 500 bp in length. In yet another embodiment, the dsRNA is about 22 bp in length.

[0060] The 21 to 23 nt RNAs described herein can be used in a variety of ways. For example, the 21 to 23 nt RNA molecules can be used to mediate RNA interference of mRNA of a gene in a cell or organism. In a specific embodiment, the 21 to 23 nt RNA is introduced into human cells or a human in order to mediate RNA interference in the cells or in cells in the individual, such as to prevent or treat a disease or undesirable condition. In this method, a gene (or genes) that cause or contribute to the disease or undesirable condition is targeted and the corresponding mRNA (the transcriptional product of the targeted gene) is degraded by RNAi. In this embodiment, an RNA of about 21 to about 23 nucleotides that targets the corresponding mRNA (the mRNA of the targeted gene) for degradation is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of the corresponding mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism. In a particular embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides. The 21 to 23 nt RNA is then isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the gene in the cell or organism. In the event that the 21-23 nt RNA is introduced into a cell in which RNAi, does not normally occur, the factors needed to mediate RNAi are introduced into such a cell or the expression of the needed factors is induced in such a cell. Alternatively, 21 to 23 nt RNA produced by other methods (e.g., chemical synthesis, recombinant DNA production) to have a composition the same as or sufficiently similar to a 21 to 23 nt RNA known to mediate RNAi can be similarly used to mediate RNAi. Such 21 to 23 nt RNAs can be altered by addition, deletion, substitution or modification of one or more nucleotides and/or can comprise non-nucleotide materials. A further embodiment of this invention is an ex vivo method of treating cells from an individual to degrade a gene(s) that causes or is associated with a disease or undesirable condition, such as leukemia or AIDS. In this embodiment, cells to be treated are obtained from the individual using known methods (e.g., phlebotomy or collection of bone marrow) and 21-23 nt RNAs that mediate degradation of the corresponding mRNA(s) are introduced into the cells, which are then re-introduced into the individual. If necessary, biochemical components needed for RNAi to occur can also be introduced into the cells.

[0061] The mRNA of any gene can be targeted for degradation using the methods of mediating interference of mRNA described herein. For example, any cellular or viral mRNA, can be targeted, and, as a result, the encoded protein (e.g., an oncoprotein, a viral protein), expression will be diminished. In addition, the mRNA of any protein associated with/causative of a disease or undesirable condition can be targeted for degradation using the methods described herein.

[0062] The present invention also relates to a method of examining the function of a gene in a cell or organism. In one embodiment, an RNA sequence of about 21 to about 23 nucleotides that targets mRNA of the gene for degradation is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of mRNA of the gene occurs. The phenotype of the cell or organism is then observed and compared to an appropriate control, thereby providing information about the function of

the gene. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract derived from Drosophila embryo under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of the mRNA of the gene occurs. The phenotype of the cell or organism is then observed and compared to an appropriate control, thereby identifying the function of the gene.

[0063] A further aspect of this invention is a method of assessing the ability of 21-23 nt RNAs to mediate RNAi and, particularly, determining which 21-23 nt RNA(s) most efficiently mediate RNAi. In one embodiment of the method, dsRNA corresponding to a sequence of an mRNA to be degraded is combined with detectably labeled (e.g., end-labeled, such as radiolabeled) mRNA and the soluble extract of this invention, thereby producing a combination. The combination is maintained under conditions under which the double-stranded RNA is processed and the mRNA is degraded. The sites of the most effective cleavage are mapped by comparing the migration of the labeled mRNA cleavage products to markers of known length. 21 mers spanning these sites are then designed and tested for their efficiency in mediating RNAi.

[0064] Alternatively, the extract of the present invention can be used to determine whether there is a particular segment or particular segments of the mRNA corresponding to a gene which are more efficiently targeted by RNAi than other regions and, thus, can be especially useful target sites. In one embodiment, dsRNA corresponding to a sequence of a gene to be degraded, labeled mRNA of the gene is combined with a soluble extract that mediates RNAi, thereby producing a combination. The resulting combination is maintained under conditions under which the dsRNA is degraded and the sites on the mRNA that are most efficiently cleaved are identified, using known methods, such as comparison to known size standards on a sequencing gel.

OVERVIEW OF EXAMPLES

[0065] Biochemical analysis of RNAi has become possible with the development of the in vitro Drosophila embryo lysate that recapitulates dsRNA-dependent silencing of gene expression described in Example 1 (Tuschl et al., Genes Dev., 13:3191-7 (1999)). In the in vitro system, dsRNA, but not sense or asRNA, targets a corresponding mRNA for degradation, yet does not affect the stability of an unrelated control mRNA. Furthermore, pre-incubation of the dsRNA in the lysate potentiates its activity for target mRNA degradation, suggesting that the dsRNA must be converted to an active form by binding proteins in the extract or by covalent modification (Tuschl et al., Genes Dev., 13:3191-7 (1999)).

[0066] The development of a cell-free system from syncytial blastoderm Drosophila embryos that recapitulates many of the features of RNAi is described herein. The interference observed in this reaction is sequence-specific, is promoted by dsRNA, but not by single-stranded RNA, functions by specific mRNA degradation, requires a minimum length of dsRNA and is most efficient with long dsRNA. Furthermore, preincubation of dsRNA potentiates its activity. These results demonstrate that RNAi is mediated by sequence specific processes in soluble reactions.

[0067] As described in Example 2, the in vitro system was used to analyze the requirements of RNAi and to determine the fate of the dsRNA and the mRNA. RNAi in vitro requires ATP, but does not require either mRNA translation or recognition of the 7-methyl-guanosine cap of the targeted mRNA. The dsRNA, but not single-stranded RNA, is processed in vitro to a population of 21-23 nt species. Deamination of adenosines within the dsRNA does not appear to be required for formation of the 21-23 nt RNAs. As described herein, the mRNA is cleaved only in the region corresponding to the sequence of the dsRNA and that the mRNA is cleaved at 21-23 nt intervals, strongly indicating that the 21-23 nt fragments from the dsRNA are targeting the cleavage of the mRNA. Furthermore, as described in Examples 3 and 4, when the 21-23 nt fragments are purified and added back to the soluble extract, they mediate RNA.

[0068] The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

Example 1

Targeted mRNA Degradation by Double-Stranded RNA in vitro Materials and Methods

[0069] RNAs

[0070] Rr-Luc mRNA consisted of the 926 nt Rr luciferase coding sequence flanked by 25 nt of 5' untranslated sequence from the pSP64 plasmid polylinker and 25 nt of 3' untranslated sequence consisting of 19 nt of pSP64 plasmid polylinker sequence followed by a 6 nt Sac I site. Pp-Luc mRNA contained the 1653 nt Pp luciferase coding sequence with a Kpn I site introduced immediately before the Pp luciferase stop codon. The Pp coding sequence was flanked by 5' untranslated sequences consisting of 21 nt of pSP64 plasmid polylinker followed by the 512 nt of the 5' untranslated region (UTR) from the Drosophila hunchback mRNA and 3' untranslated sequences consisting of the 562 nt hunchback 3' UTR followed by a 6 nt Sac I site. The hunchback 3' UTR sequences used contained six G-to-U mutations that disrupt function of the Nanos Response Elements in vivo and in vitro. Both reporter mRNAs terminated in a 25 nt poly(A) tail encoded in the transcribed plasmid. For both Rr-Luc and Pp-Luc mRNAs, the transcripts were generated by run-off transcription from plasmid templates cleaved at an Nsi I site that immediately followed the 25 nt encoded poly(A) tail. To ensure that the transcripts ended with a poly(A) tail, the Nsi I-cleaved transcription templates were resected with T4 DNA Polymerase in the presence of dNTPs. The SP6 mMessage mMachine kit (Ambion) was used for in vitro transcription. Using this kit, about 80% of the resulting transcripts are 7-methyl guanosine capped. ^{32}P-radiolabeling was accomplished by including α-^{32}P-UTP in the transcription reaction.

[0071] For Pp-Luc, ss, as, and dsRNA corresponded to positions 93 to 597 relative to the start of translation, yielding a 505 bp dsRNA. For Rr-Luc, ss, as, and dsRNA corresponded to positions 118 to 618 relative to the start of translation, yielding a 501 bp dsRNA. The Drosophila nanos competitor dsRNA corresponded to positions 122 to 629 relative to the start of translation, yielding a 508 bp dsRNA.

ssRNA, asRNA, and dsRNA (diagrammed in **FIG. 1**) were transcribed in vitro with T7 RNA polymerase from templates generated by the polymerase chain reaction. After gel purification of the T7 RNA transcripts, residual DNA template was removed by treatment with RQ1 DNase (Promega). The RNA was then extracted with phenol and chloroform, and then precipitated and dissolved in water.

[0072] RNA Annealing and Native Gel Electrophoresis.

[0073] ssRNA and asRNA (0.5 μM) in 10 mM Tris-HCl (pH 7.5) with 20 mM NaCl were heated to 95° C. for 1 min then cooled and annealed at room temperature for 12 to 16 h. The RNAs were precipitated and resuspended in lysis buffer (below). To monitor annealing, RNAs were electrophoresed in a 2% agarose gel in TBE buffer and stained with ethidium bromide (Sambrook et al., Molecular Cloning. Cold Spring Harbor Laboratory Press, Plainview, N.Y. (1989)).

[0074] Lysate Preparation

[0075] Zero- to two-hour old embryos from Oregon R flies were collected on yeasted molasses agar at 25° C. Embryos were dechorionated for 4 to 5 min in 50% (v/v) bleach, washed with water, blotted dry, and transferred to a chilled Potter-Elvehjem tissue grinder (Kontes). Embryos were lysed at 4° C. in one ml of lysis buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) containing 5 mM dithiothreitol (DTT) and 1 mg/ml Pefabloc SC (Boehringer-Mannheim) per gram of damp embryos. The lysate was centrifuged for 25 min at 14,500 × g at 4° C., and the supernatant flash frozen in aliquots in liquid nitrogen and stored at −80° C.

[0076] Reaction Conditions

[0077] Lysate preparation and reaction conditions were derived from those described by Hussain and Leibowitz (Hussain and Leibowitz, Gene 46:13-23 (1986)). Reactions contained 50% (v/v) lysate, mRNAs (10 to 50 pM final concentration), and 10% (v/v) lysis buffer containing the ssRNA, asRNA, or dsRNA (10 nM final concentration). Each reaction also contained 10 mM creatine phosphate, 10 μg/ml creatine phosphokinase, 100 μM GTP, 100 μM UTP, 100 μM CTP, 500 μM ATP, 5 μM DTT, 0.1 U/mL RNasin (Promega), and 100 μM of each amino acid. The final concentration of potassium acetate was adjusted to 100 mM. For standard conditions, the reactions were assembled on ice and then pre-incubated at 25° C. for 10 min before adding mRNA. After adding mRNAs, the incubation was continued for an additional 60 min. The 10 min preincubation step was omitted for the experiments in FIGS. **3**A-3C and **5**A-5C. Reactions were quenched with four volumes of 1.25× Passive Lysis Buffer (Promega). Pp and Rr luciferase activity was detected in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory) using the Dual-Luciferase Reporter Assay System (Promega).

[0078] RNA Stability

[0079] Reactions with ^{32}P-radiolabeled mRNA were quenched by the addition of 40 volumes of 2× PK buffer (200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% w/v sodium dodecyl sulfate). Proteinase K (E.M. Merck; dissolved in water) was added to a final concentration of 465 μg/ml. The reactions were then incubated for 15 min at 65° C., extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with an equal volume of isopropanol. Reactions were analyzed by electrophoresis in a formaldehyde/agarose (0.8% w/v) gel (Sambrook et al., Molecular Cloning. Cold Spring Harbor Laboratory Press, Plainview, N.Y. (1989)). Radioactivity was detected by exposing the agarose gel [dried under vacuum onto Nytran Plus membrane (Amersham)] to an image plate (Fujix) and quantified using a Fujix Bas 2000 and Image Gauge 3.0 (Fujix) software.

[0080] Commercial Lysates

[0081] Untreated rabbit reticulocyte lysate (Ambion) and wheat germ extract (Ambion) reactions were assembled according to the manufacturer's directions. dsRNA was incubated in the lysate at 27° C. (wheat germ) or 30° C. (reticulocyte lysate) for 10 min prior to the addition of mRNAs.

[0082] Results and Discussion

[0083] To evaluate if dsRNA could specifically block gene expression in vitro, reporter mRNAs derived from two different luciferase genes that are unrelated both in sequence and in luciferin substrate specificity were used: *Renilla reniformis* (sea pansy) luciferase (Rr-Luc) and Photuris pennsylvanica (firefly) luciferase (Pp-Luc). dsRNA generated from one gene was used to target that luciferase mRNA whereas the other luciferase mRNA was an internal control co-translated in the same reaction. dsRNAs of approximately 500 bp were prepared by transcription of polymerase-chain reaction products from the Rr-Luc and Pp-Luc genes. Each dsRNA began ~100 bp downstream of the start of translation (**FIG. 1**). Sense (ss) and anti-sense (as) RNA were transcribed in vitro and annealed to each other to produce the dsRNA. Native gel electrophoresis of the individual Rr 501 and Pp 505 nt as RNA and ssRNA used to form the Rr and Pp dsRNAs was preformed. The ssRNA, asRNA, and dsRNAs were each tested for their ability to block specifically expression of their cognate mRNA but not the expression of the unrelated internal control mRNA.

[0084] The ssRNA, asRNA, or dsRNA was incubated for 10 min in a reaction containing Drosophila embryo lysate, then both Pp-Luc and Rr-Luc mRNAs were added and the incubation continued for an additional 60 min. The Drosophila embryo lysate efficiently translates exogenously transcribed mRNA under the conditions used. The amounts of Pp-Luc and Rr-Luc enzyme activities were measured and were used to calculate ratios of either Pp-Luc/Rr-Luc (**FIG. 2A**) or Rr-Luc/Pp-Luc (**FIG. 2B**). To facilitate comparison of different experiments, the ratios from each experiment were normalized to the ratio observed for a control in which buffer was added to the reaction in place of ssRNA, asRNA, or dsRNA.

[0085] **FIG. 2A** shows that a 10 nM concentration of the 505 bp dsRNA identical to a portion of the sequence of the Pp-Luc gene specifically inhibited expression of the Pp-Luc mRNA but did not affect expression of the Rr-Luc internal control. Neither ssRNA nor asRNA affected expression of Pp-Luc or the Rr-Luc internal control. Thus, Pp-Luc expression was specifically inhibited by its cognate dsRNA. Conversely, a 10 nM concentration of the 501 bp dsRNA directed against the Rr-Luc mRNA specifically inhibited Rr-Luc expression but not that of the Pp-Luc internal control (**FIG. 2B**). Again, comparable levels of ssRNA or asRNA

had little or no effect on expression of either reporter mRNA. On average, dsRNA reduced specific luciferase expression by 70% in these experiments, in which luciferase activity was measured after 1 h incubation. In other experiments in which the translational capacity of the reaction was replenished by the addition of fresh lysate and reaction components, a further reduction in targeted luciferase activity relative to the internal control was observed.

[0086] The ability of dsRNA but not asRNA to inhibit gene expression in these lysates is not merely a consequence of the greater stability of the dsRNA (half-life about 2 h) relative to the single-stranded RNAs (half-life ~10 min). ssRNA and asRNA transcribed with a 7-methyl guanosine cap were as stable in the lysate as uncapped dsRNA, but do not inhibit gene expression. In contrast, dsRNA formed from the capped ssRNA and asRNA specifically blocks expression of the targeted mRNA.

[0087] Effective RNAi in Drosophila requires the injection of about 0.2 fmol of dsRNA into a syncytial blastoderm embryo (Kennerdell and Carthew, Cell 95:1017-1026 (1998); Carthew, www1.pitt.edu/~carthew/manual/RNAi_Protocol.html (1999)). Since the average volume of a Drosophila embryo is approximately 7.3 nl, this corresponds to an intracellular concentration of about 25 nM (Mazur et al., Cryobiology 25:543-544 (1988)). Gene expression in the Drosophila lysate was inhibited by a comparable concentration of dsRNA (10 nM), but lowering the dsRNA concentration ten-fold decreased the amount of specific interference. Ten nanomolar dsRNA corresponds to a 200-fold excess of dsRNA over target mRNA added to the lysate. To test if this excess of dsRNA might reflect a time- and/or concentration-dependent step in which the input dsRNA was converted to a form active for gene-specific interference, the effect of preincubation of the dsRNA on its ability to inhibit expression of its cognate mRNA was examined. Because the translational capacity of the lysates is significantly reduced after 30 min of incubation at 25° C. (unpublished observations), it was desired to ensure that all factors necessary for RNAi remained active throughout the pre-incubation period. Therefore, every 30 min, a reaction containing dsRNA and lysate was mixed with a fresh reaction containing unincubated lysate (**FIG. 3A**). After six successive serial transfers spanning 3 hours of preincubation, the dsRNA, now diluted 64-fold relative to its original concentration, was incubated with lysate and 50 pM of target mRNA for 60 min. Finally, the Pp-Luc and Rr-Luc enzyme levels were measured. For comparison, the input amount of dsRNA (10 nM) was diluted 32-fold in buffer, and its capacity to generate gene-specific dsRNA interference in the absence of any preincubation step was assessed.

[0088] The preincubation of the dsRNA in lysate significantly potentiated its capacity to inhibit specific gene expression. Whereas the dsRNA diluted 32-fold showed no effect, the preincubated dsRNA was, within experimental error, as potent as undiluted dsRNA, despite having undergone a 64-fold dilution. Potentiation of the dsRNA by preincubation was observed for dsRNAs targeting both the Pp-Luc mRNA (**FIG. 3B**) and the Rr-Luc mRNA (**FIG. 3C**). Taking into account the 64-fold dilution, the activation conferred by preincubation allowed a 156 pM concentration of dsRNA to inhibit 50 pM target mRNA. Further, dilution of the "activated" dsRNA may be effective but has not been tested. We note that although both dsRNAs tested were activated by the preincubation procedure, each fully retained its specificity to interfere with expression only of the mRNA to which it is homologous. Further study of the reactions may provide a route to identifying the mechanism of dsRNA potentiation.

[0089] One possible explanation for the observation that preincubation of the dsRNA enhances its capacity to inhibit gene expression in these lysates is that specific factors either modify and/or associate with the dsRNA. Accordingly, the addition of increasing amounts of dsRNA to the reaction might titrate such factors and decrease the amount of gene-specific interference caused by a second dsRNA of unrelated sequence. For both Pp-Luc mRNA and Rr-Luc mRNA, addition of increasing concentrations of the unrelated Drosophila nanos dsRNA to the reaction decreased the amount of gene-specific interference caused by dsRNA targeting the reporter mRNA (**FIG. 4**). None of the tested concentrations of nanos dsRNA affected the levels of translation of the untargeted mRNA, demonstrating that the nanos dsRNA specifically titrated factors involved in gene-specific interference and not components of the translational machinery. The limiting factor(s) was titrated by addition of approximately 1000 nM dsRNA, a 200-fold excess over the 5 nM of dsRNA used to produce specific interference.

[0090] Interference in vitro might reflect either a specific inhibition of mRNA translation or the targeted destruction of the specific mRNA. To distinguish these two possibilities, the fates of the Pp-Luc and Rr-Luc mRNAs were examined directly using ^{32}P-radiolabeled substrates. Stability of 10 nM Pp-Luc mRNA or Rr-Luc mRNA incubated in lysate with either buffer or 505 bp Pp-dsRNA (10 nM). Samples were deproteinized after the indicated times and the ^{32}P-radiolabeled mRNAs were then resolved by denaturing gel electrophoresis. In the absence of dsRNA, both the Pp-Luc and Rr-Luc mRNAs were stable in the lysates, with ~75% of the input mRNA remaining after 3 h of incubation. (About 25% of the input mRNA is rapidly degraded in the reaction and likely represents uncapped mRNA generated by the in vitro transcription process.) In the presence of dsRNA (10 nM, 505 bp) targeting the Pp-Luc mRNA, less than 15% of the Pp-Luc mRNA remained after 3 h (**FIG. 5A**). As expected, the Rr-Luc mRNA remained stable in the presence of the dsRNA targeting Pp-Luc mRNA. Conversely, dsRNA (10 nM, 501 bp) targeting the Rr-Luc mRNA caused the destruction of the Rr-Luc mRNA but had no effect on the stability of Pp-Luc mRNA (**FIG. 5B**). Thus, the dsRNA specifically caused accelerated decay of the mRNA to which it is homologous with no effect on the stability of the unrelated control mRNA. This finding indicates that in vivo, at least in Drosophila, the effect of dsRNA is to directly destabilize the target mRNA, not to change the subcellular localization of the mRNA, for example, by causing it to be specifically retained in the nucleus, resulting in non-specific degradation.

[0091] These results are consistent with the observation that RNAi leads to reduced cytoplasmic mRNA levels in vivo, as measured by in situ hybridization (Montgomery et al., Proc. Natl. Acad. Sci. USA 95:15502-15507 (1998)) and Northern blotting (Ngo et al., Proc. Natl. Acad. Sci. USA 95:14687-14692 (1998)). Northern blot analyses in trypanosomes and hydra suggest that dsRNA typically decreases mRNA levels by less than 90% (Ngo et al., Proc. Natl. Acad. Sci. USA 95:14687-14692 (1998); Lohmann et al., Dev.

Biol. 214:211-214 (1999)). The data presented here show that in vitro mRNA levels are reduced 65 to 85% after three hours incubation, an effect comparable with observations in vivo. They also agree with the finding that RNAi in *C. elegans* is post-transcriptional (Montgomery et al., Proc. Natl. Acad. Sci. USA 95:15502-15507 (1998)). The simplest explanation for the specific effects on protein synthesis is that it reflects the accelerated rate of RNA decay. However, the results do not exclude independent but specific effects on translation as well as stability.

[0092] In vivo, RNAi appears to require a minimum length of dsRNA (Ngo et al., Proc. Natl. Acad. Sci., USA, 95:14687-14692 (1998)). The ability of RNA duplexes of lengths 49 bp, 149 bp, 505 bp, and 997 bp (diagrammed in **FIG. 1**) to target the degradation of the Pp-Luc mRNA in vitro was assessed. In good agreement with in vivo observations, the 49 bp dsRNA was ineffective in vitro, while the 149 bp dsRNA enhanced mRNA decay only slightly, and both the 505 and 997 bp dsRNAs caused robust mRNA degradation (**FIG. 5C**). 50bp dsRNA targeting other portions of the mRNA cause detectable mRNA degradation, though not as robust as that seen for 500bp dsRNA. Thus, although some short dsRNA do not mediate RNAi, others of approximately the same length, but different composition, will be able to do so.

[0093] Whether the gene-specific interference observed in Drosophila lysates was a general property of cell-free translation systems was examined. The effects of dsRNAs on expression of Pp-Luc and Rr-Luc mRNA were examined in commercially available wheat germ extracts and rabbit reticulocyte lysates. There was no effect of addition of 10 nM of either ssRNA, asRNA, or dsRNA on the expression of either mRNA reporter in wheat germ extracts. In contrast, the addition of 10 nM of dsRNA to the rabbit reticulocyte lysate caused a profound and rapid, non-specific decrease in mRNA stability. For example, addition of Rr-Luc dsRNA caused degradation of both Rr-Luc and Pp-Luc mRNAs within 15 min. The same non-specific effect was observed upon addition of Pp-Luc dsRNA. The non-specific destruction of mRNA induced by the addition of dsRNA to the rabbit reticulocyte lysate presumably reflects the previously observed activation of RNase L by dsRNA (Clemens and Williams, Cell 13:565-572 (1978); Williams et al., Nucleic Acids Res. 6:1335-1350 (1979); Zhou et al., Cell 72:753-765 (1993); Matthews, Interactions between Viruses and the Cellular Machinery for Protein Synthesis. In Translational Control (eds. J. Hershey, M. Mathews and N. Sonenberg), pp. 505-548. Cold Spring Harbor Laboratory Press, Plainview, N.Y. (1996)). Mouse cell lines lacking dsRNA-induced anti-viral pathways have recently been described (Zhou et al., Virology 258:435-440 (1999)) and may be useful in the search for mammalian RNAi. Although RNAi is known to exist in some mammalian cells (Wianny and Zernicka-Goetz Nat. Cell Biol. 2: 70-75 (2000)), in many mammalian cell types its presence is likely obscured by the rapid induction by dsRNA of non-specific anti-viral responses.

[0094] dsRNA-targeted destruction of specific mRNA is characteristic of RNAi, which has been observed in vivo in many organisms, including Drosophila. The system described above recapitulates in a reaction in vitro many aspects of RNAi. The targeted mRNA is specifically degraded whereas unrelated control mRNAs present in the same solution are not affected. The process is most efficient with dsRNAs greater than 150 bp in length. The dsRNA-specific degradation reaction in vitro is probably general to many, if not all, mRNAs since it was observed using two unrelated genes.

[0095] The magnitude of the effects on mRNA stability in vitro described herein are comparable with those reported in vivo (Ngo et al., Proc. Natl. Acad. Sci., USA, 95:14687-14692 (1998); Lohmann et al., Dev. Biol., 214:211-214 (1999). However, the reaction in vitro requires an excess of dsRNA relative to mRNA. In contrast, a few molecules of dsRNA per cell can inhibit gene expression in vivo (Fire et al., Nature, 391: 806-811 (1998); Kennerdell and Carthew, Cell, 95:1017-1026 (1998)). The difference between the stoichiometry of dsRNA to target mRNA in vivo and in vitro should not be surprising in that most in vitro reactions are less efficient than their corresponding in vivo processes. Interestringly, incubation of the dsRNA in the lysate greatly potentiated its activity for RNAi, indicating that it is either modified or becomes associated with other factors or both. Perhaps a small number of molecules is effective in inhibiting the targeted mRNA in vivo because the injected dsRNA has been activated by a process similar to that reported here for RNAi in Drosophila lysates.

Example 2

Double-Stranded RNA Directs the ATP-Dependent Cleavage of mRNA at 21 to 23 Nucleotide Intervals

[0096] Methods and Material

[0097] In vitro RNAi

[0098] In vitro RNAi reactions and lysate preparation were as described in Example 1 (Tuschl et al., Genes Dev., 13:3191-7 (1999)) except that the reaction contained 0.03 g/ml creatine kinase, 25 μM creatine phosphate (Fluka), and 1 mM ATP. Creatine phosphate was freshly dissolved at 500 mM in water for each experiment. GTP was omitted from the reactions, except in **FIGS. 2 and 3**.

[0099] RNA Synthesis.

[0100] Pp-luc and Rr-luc mRNAs and Pp- and Rr-dsRNAs (including dsRNA 'B' in **FIG. 6**) were synthesized by in vitro transcription as described previously (Tuschl et al., Genes Dev., 13:3191-7 (1999)). To generate transcription templates for dsRNA 'C' the 5' sense RNA primer was gcgtaatacgactcactataGAACAAAGGAAACGGATGAT (SEQ ID NO: 2) and the 3' sense RNA primer was GAAGAAGTTATTCTCCAAAA (SEQ ID NO: 3); the 5' asRNA primer was gcgtaatacgactcactataGAAGAAGTTATTCTCCAAAA (SEQ ID NO: 4) and the 3' asRNA primer was GAACAAAGGAAACGGATGAT (SEQ ID NO: 5). For dsRNA 'A' the 5' sense RNA primer was gcgtaatacgactcactataGTAGCGCGGTGTATTATACC (SEQ ID NO: 6) and the 3' sense RNA primer was GTACAACGTCAGGTTTACCA (SEQ ID NO: 7); the 5' asRNA primer was gcgtaatacgactcactataGTACAACGTCAGGTTTACCA (SEQ ID NO: 8) and the 3' asRNA primer was GTAGCGCGGTGTATTATACC (SEQ ID NO: 9) (lowercase, T7 promoter sequence).

[0101] mRNAs were 5'-end-labeled using guanylyl transferase (Gibco/BRL), S-adenosyl methionine (Sigma), and

α-^{32}P-GTP (3000 Ci/mmol; New England Nuclear) according to the manufacturer's directions. Radiolabeled RNAs were purified by poly(A) selection using the Poly(A) Tract III kit (Promega). Nonradioactive 7-methyl-guanosine- and adenosine-capped RNAs were synthesized in in vitro transcription reactions with a 5-fold excess of 7-methyl-G(5')ppp(5')G or A(5')ppp(5')G relative to GTP. Cap analogs were purchased from New England Biolabs.

[0102] ATP Depletion and Protein Synthesis Inhibition

[0103] ATP was depleted by incubating the lysate for 10 minutes at 25° C. with 2 mM glucose and 0.1 U/ml hexokinase (Sigma). Protein synthesis inhibitors were purchased from Sigma and dissolved in absolute ethanol as 250-fold concentrated stocks. The final concentrations of inhibitors in the reaction were: anisomycin, 53 mg/ml; cycloheximide, 100 mg/ml; chloramphenicol, 100 mg/ml. Relative protein synthesis was determined by measuring the activity of Rr luciferase protein produced by translation of the Rr-luc mRNA in the RNAi reaction after 1 hour as described previously (Tuschl et al., Genes Dev., 13:3191-7 (1999)).

[0104] Analysis of dsRNA Processing

[0105] Internally α-^{32}P-ATP-labeled dsRNAs (505 bp Pp-luc or 501 Rr-luc) or 7-methyl-guanosine-capped Rr-luc antisense RNA (501 nt) were incubated at 5 nM final concentration in the presence or absence of unlabeled mRNAs in Drosophila lysate for 2 hours in standard conditions. Reactions were stopped by the addition of 2× proteinase K buffer and deproteinized as described previously (Tuschl et al., Genes Dev., 13:3191-3197 (1999)). Products were analyzed by electrophoresis in 15% or 18% polyacrylamide sequencing gels. Length standards were generated by complete RNase Ti digestion of α-^{32}P-ATP-labeled 501 nt Rr-luc sense RNA and asRNA.

[0106] For analysis of mRNA cleavage, 5'-^{32}P-radiolabeled mRNA (described above) was incubated with dsRNA as described previously (Tuschl et al., Genes Dev., 13:3191-3197 (1999)) and analyzed by electrophoresis in 5% **(FIG. 5B)** and 6% **(FIG. 6C)** polyacrylamide sequencing gels. Length standards included commercially available RNA size standards (FMC Bioproducts) radiolabeled with guanylyl transferase as described above and partial base hydrolysis and RNase Ti ladders generated from the 5'-radiolabeled mRNA.

[0107] Deamination Assay

[0108] Internally α-^{32}P-ATP-labeled dsRNAs (5 nM) were incubated in Drosophila lysate for 2 hours at standard conditions. After deproteinization, samples were run on 12% sequencing gels to separate full-length dsRNAs from the 21-23 nt products. RNAs were eluted from the gel slices in 0.3 M NaCl overnight, ethanol-precipitated, collected by centrifugation, and redissolved in 20 μl water. The RNA was hydrolyzed into nucleoside 5 -phosphates with nuclease P1 (10 μl reaction containing 8 μl RNA in water, 30 mM KOAc pH 5.3, 10 mM $ZnSO_4$, 10 μg or 3 units nuclease P1, 3 hours, 50° C). Samples (1 ml) were co-spotted with non-radioactive 5 -mononucleotides [0.05 O.D. units (A_{260}) of pA, pC, pG, pI, and pU] on cellulose HPTLC plates (EM Merck) and separated in the first dimension in isobutyric acid/25% ammonia/water (66/1/33, v/v/v) and in the second dimension in 0.1M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol (100/60/2, v/w/v; Silberklang et al., 1979). Migration of the non-radioactive internal standards was determined by UV-shadowing.

[0109] Results and Discussion

[0110] RNAi Requires ATP

[0111] As described in Example 1, Drosophila embryo lysates faithfully recapitulate RNAi (Tuschl et al., Genes Dev., 13:3191-7 (1999)). Previously, dsRNA-mediated gene silencing was monitored by measuring the synthesis of luciferase protein from the targeted mRNA. Thus, these RNAi reactions contained an ATP-regenerating system, needed for the efficient translation of the mRNA. To test if ATP was, in fact, required for RNAi, the lysates were depleted for ATP by treatment with hexokinase and glucose, which converts ATP to ADP, and RNAi was monitored directly by following the fate of ^{32}P-radiolabeled *Renilla reniformis* luciferase (Rr-luc) mRNA **(FIG. 6)**. Treatment with hexokinase and glucose reduced the endogenous ATP level in the lysate from 250 μM to below 10 μM. ATP regeneration required both exogenous creatine phosphate and creatine kinase, which acts to transfer a high-energy phosphate from creatine phosphate to ADP. When ATP-depleted extracts were supplemented with either creatine phosphate or creatine kinase separately, no RNAi was observed. Therefore, RNAi requires ATP in vitro. When ATP, creatine phosphate, and creatine kinase were all added together to reactions containing the ATP-depleted lysate, dsRNA-dependent degradation of the Rr-luc mRNA was restored **(FIG. 6)**. The addition of exogenous ATP was not required for efficient RNAi in the depleted lysate, provided that both creatine phosphate and creatine kinase were present, demonstrating that the endogenous concentration (250 mM) of adenosine nucleotide is sufficient to support RNAi. RNAi with a *Photinus pyralis* luciferase (Pp-luc) mRNA was also ATP-dependent.

[0112] The stability of the Rr-luc mRNA in the absence of Rr-dsRNA was reduced in ATP-depleted lysates relative to that observed when the energy regenerating system was included, but decay of the mRNA under these conditions did not display the rapid decay kinetics characteristic of RNAi in vitro, nor did it generate the stable mRNA cleavage products characteristic of dsRNA-directed RNAi. These experiments do not establish if the ATP requirement for RNAi is direct, implicating ATP in one or more steps in the RNAi mechanism, or indirect, reflecting a role for ATP in maintaining high concentrations of another nucleoside triphosphate in the lysate.

[0113] Translation Is Not Required for RNAi In Vitro

[0114] The requirement for ATP suggested that RNAi might be coupled to mRNA translation, a highly energy-dependent process. To test this possibility, various inhibitors of protein synthesis were added to the reaction by preparing a denaturing agarose-gel analysis of 5'-32P-radiolabeled Pp-luc mRNA after incubation for indicated times in a standard RNAi reaction with and without protein synthesis inhibitors. The eukaryotic translation inhibitors anisomycin, an inhibitor of initial peptide bond formation, cycloheximide, an inhibitor of peptide chain elongation, and puromycin, a tRNA mimic which causes premature termination of translation (Cundliffe, Antibiotic Inhibitors of Ribosome Function. In The Molecular Basis of Antibiotic Action, E.

Gale, E. Cundliffe, P. Reynolds, M. Richmond and M. Warning, eds. (New York: Wiley), pp. 402-547. (1981)) were tested. Each of these inhibitors reduced protein synthesis in the Drosophila lysate by more than 1,900-fold (**FIG. 7A**). In contrast, chloramphenicol, an inhibitor of Drosophila mitochondrial protein synthesis (Page and Orr-Weaver, Dev. Biol., 183:195-207 (1997)), had no effect on translation in the lysates (**FIG. 7A**). Despite the presence of anisomycin, cycloheximide, or chloramphenicol, RNAi proceeded at normal efficiency. Puromycin also did not perturb efficient RNAi. Thus, protein synthesis is not required for RNAi in vitro.

[0115] Translational initiation is an ATP-dependent process that involves recognition of the 7-methyl guanosine cap of the mRNA (Kozak, Gene, 234:187-208 (1999); Merrick and Hershey, The Pathway and Mechanism of Eukaryotic Protein Synthesis. In Translational Control, J. Hershey, M. Mathews and N. Sonenberg, eds. (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press), pp. 31-69 (1996)). The Drosophila lysate used to support RNAi in vitro also recapitulates the cap-dependence of translation; Pp-luc mRNA with a 7-methyl-guanosine cap was translated greater than ten-fold more efficiently than was the same mRNA with an A(5')ppp(5')G cap (FIG. 7B). Both RNAs were equally stable in the Drosophila lysate, showing that this difference in efficiency cannot be merely explained by more rapid decay of the mRNA with an adenosine cap (see also Gebauer et al., EMBO J., 18:6146-54 (1999)). Although the translational machinery can discriminate between Pp-luc mRNAs with 7-methyl-guanosine and adenosine caps, the two mRNAs were equally susceptible to RNAi in the presence of Pp-dsRNA (**FIG. 7C**). These results suggest that steps in cap recognition are not involved in RNAi.

[0116] dsRNA Is Processed to 21-23 nt Species

[0117] RNAs 25 nt in length are generated from both the sense and anti-sense strands of genes undergoing post-transcriptional gene silencing in plants (Hamilton and Baulcombe, Science, 286:950-2 (1999)). Denaturing acrylamide-gel analysis of the products formed in a two-hour incubation of uniformly ^{32}P-radiolabeled dsRNAs and capped asRNA in lysate under standard RNAi conditions, in the presence or absence of target mRNAs. It was found that dsRNA is also processed to small RNA fragments. When incubated in lysate, approximately 15% of the input radioactivity of both the 501 bp Rr-dsRNA and the 505 bp Pp-dsRNA appeared in 21 to 23 nt RNA fragments. Because the dsRNAs are more than 500 bp in length, the 15% yield of fragments implies that multiple 21-23 nt RNAs are produced from each full-length dsRNA molecule. No other stable products were detected. The small RNA species were produced from dsRNAs in which both strands were uniformly ^{32}P-radiolabeled. Formation of the 21-23 nt RNAs from the dsRNA did not require the presence of the corresponding mRNA, demonstrating that the small RNA species is generated by processing of the dsRNA, rather than as a product of dsRNA-targeted mRNA degradation. It was noted that 22 nucleotides corresponds to two turns of an A-form RNA-RNA helix.

[0118] When dsRNAs radiolabeled within either the sense or the anti-sense strand were incubated with lysate in a standard RNAi reaction, 21-23 nt RNAs were generated with comparable efficiency. These data support the idea that the 21-23 nt RNAs are generated by symmetric processing of the dsRNA. A variety of data support the idea that the 21-23 nt RNA is efficiently generated only from dsRNA and is not the consequence of an interaction between single-stranded RNA and the dsRNA. First, a ^{32}P-radiolabeled 505 nt Pp-luc sense RNA or asRNA was not efficiently converted to the 21-23 nt product when it was incubated with 5 nM nonradioactive 505 bp Pp-dsRNA. Second, in the absence of mRNA, a 501 nt 7-methyl-guanosine-capped Rr-asRNA produced only a barely detectable amount of 21-23 nt RNA (capped single-stranded RNAs are as stable in the lysate as dsRNA, Tuschl et al., Genes Dev., 13:3191-7(1999)), probably due to a small amount of dsRNA contaminating the anti-sense preparation. However, when Rr-luc mRNA was included in the reaction with the ^{32}P-radiolabeled, capped Rr-asRNA, a small amount of 21-23 nt product was generated, corresponding to 4% of the amount of 21-23 nt RNA produced from an equimolar amount of Rr-dsRNA. This result is unlikely to reflect the presence of contaminating dsRNA in the Rr-asRNA preparation, since significantly more product was generated from the asRNA in the presence of the Rr-luc mRNA than in the absence. Instead, the data suggest that asRNA can interact with the complementary mRNA sequences to form dsRNA in the reaction and that the resulting dsRNA is subsequently processed to the small RNA species. Rr-asRNA can support a low level of bona fide RNAi in vitro (see below), consistent with this explanation.

[0119] It was next asked if production of the 21-23 nt RNAs from dsRNA required ATP. When the 505 bp Pp-dsRNA was incubated in a lysate depleted for ATP by treatment with hexokinase and glucose, 21-23 nt RNA was produced, albeit 6 times slower than when ATP was regenerated in the depleted lysate by the inclusion of creatine kinase and creatine phosphate. Therefore, ATP may not be required for production of the 21-23 nt RNA species, but may instead simply enhance its formation. Alternatively, ATP may be required for processing of the dsRNA, but at a concentration less than that remaining after hexokinase treatment. The molecular basis for the slower mobility of the small RNA fragments generated in the ATP-depleted lysate is not understood.

[0120] Wagner and Sun (Wagner and Sun, Nature, 391:744-745 (1998)) and Sharp (Sharp, Genes Dev., 13:139-41 (1999)) have speculated that the requirement for dsRNA in gene silencing by RNAi reflects the involvement of a dsRNA-specific adenosine deaminase in the process. dsRNA adenosine deaminases unwind dsRNA by converting adenosine to inosine, which does not base-pair with uracil. dsRNA adenosine deaminases function in the post-transcriptional editing of mRNA (for review see Bass, Trends Biochem. Sci., 22:157-62 (1997)). To test for the involvement of dsRNA adenosine deaminase in RNAi, the degree of conversion of adenosine to inosine in the 501 bp Rr-luc and 505 bp Pp-luc dsRNAs after incubation with Drosophila embryo lysate in a standard in vitro RNAi reaction was examined. Adenosine deamination in full-length dsRNA and the 21-23 nt RNA species was assessed by two-dimensional thin-layer chromatography. Inorganic phosphate (P_i) was produced by the degradation of mononucleotides by phosphatases that contaminate commercially available nuclease P1 (Auxilien et al., J. Mol. Biol., 262:437-458 (1996)). The degree of adenosine deamination in the 21-23 nt species was also determined. The full-length dsRNA radiolabeled with [^{32}P]-adenosine was incubated in the lysate, and both the

full-length dsRNA and the 21-23 nt RNA products were purified from a denaturing acrylarnide gel, cleaved to mononucleotides with nuclease P1, and analyzed by two-dimensional thin-layer chromatography.

[0121] A significant fraction of the adenosines in the full-length dsRNA were converted to inosine after 2 hours (3.1% and 5.6% conversion for Pp-luc and Rr-luc dsRNAs, respectively). In contrast, only 0.4% (Pp-dsRNA) or 0.7% (Rr-dsRNA) of the adenosines in the 21-23 nt species were deaminated. These data imply that fewer than 1 in 27 molecules of the 21-23 nt RNA species contain an inosine. Therefore, it is unlikely that dsRNA-dependent adenosine deamination within the 21-23 nt species is required for its production. asRNA Generates a Small Amount of RNAi in vitro When mRNA was ^{32}P-radiolabeled within the 5'-7-methyl-guanosine cap, stable 5' decay products accumulated during the RNAi reaction. Such stable 5' decay products were observed for both the Pp-luc and Rr-luc mRNAs when they were incubated with their cognate dsRNAs. Previously, it was reported that efficient RNAi does not occur when asRNA is used in place of dsRNA (Tuschl et al., Genes Dev., 13:3191-7 (1999)). Nevertheless, mRNA was measurably less stable when incubated with asRNA than with buffer (**FIGS. 8A and 8B**). This was particularly evident for the Rr-luc mRNA: approximately 90% of the RNA remained intact after a 3-hour incubation in lysate, but only 50% when asRNA was added. Less than 5% remained when dsRNA was added. Interestingly, the decrease in mRNA stability caused by asRNA was accompanied by the formation of a small amount of the stable 5'-decay products characteristic of the RNAi reaction with dsRNA. This finding parallels the observation that a small amount of 21-23 nt product formed from the asRNA when it was incubated with the mRNA (see above) and lends strength to the idea that asRNA can enter the RNAi pathway, albeit inefficiently.

[0122] mRNA Cleavage Sites Are Determined by the Sequence of the dsRNA

[0123] The sites of mRNA cleavage were examined using three different dsRNAs, 'A,''B,' and 'C,' displaced along the Rr-luc sequence by approximately 100 nts. Denaturing acrylamide-gel analysis of the stable, 5'-cleavage products produced after incubation of the Rr-luc mRNA for the indicated times with each of the three dsRNAs, 'A,''B,' and 'C,' or with buffer (Ø) was performed. The positions of these relative to the Rr-luc mRNA sequence are shown in **FIG. 9**. Each of the three dsRNAs was incubated in a standard RNAi reaction with Rr-luc mRNA ^{32}P-radiolabeled within the 5'-cap. In the absence of dsRNA, no stable 5'-cleavage products were detected for the mRNA, even after 3 hours of incubation in lysate. In contrast, after a 20-minute incubation, each of the three dsRNAs produced a ladder of bands corresponding to a set of mRNA cleavage products characteristic for that particular dsRNA. For each dsRNA, the stable, 5' mRNA cleavage products were restricted to the region of the Rr-luc mRNA that corresponded to the dsRNA (**FIGS. 9 and 10**). For dsRNA 'A,' the lengths of the 5' cleavage products ranged from 236 to just under ~750 nt; dsRNA 'A' spans nucleotides 233 to 729 of the Rr-luc mRNA. Incubation of the mRNA with dsRNA 'B' produced mRNA 5'-cleavage products ranging in length from 150 to ~600 nt; dsRNA 'B' spans nucleotides 143 to 644 of the mRNA. Finally, dsRNA 'C' produced mRNA cleavage products from 66 to ~500 nt in length. This dsRNA spans nucleotides 50 to 569 of the Rr-luc mRNA. Therefore, the dsRNA not only provides specificity for the RNAi reaction, selecting which mRNA from the total cellular mRNA pool will be degraded, but also determines the precise positions of cleavage along the mRNA sequence.

[0124] The mRNA Is Cleaved at 21-23 Nucleotide Intervals

[0125] To gain further insight into the mechanism of RNAi, the positions of several mRNA cleavage sites for each of the three dsRNAs were mapped (**FIG. 10**). High resolution denaturing acrylamide-gel analysis of a subset of the 5'-cleavage products described above was performed. Remarkably, most of the cleavages occurred at 21-23 nt intervals (**FIG. 10**). This spacing is especially striking in light of our observation that the dsRNA is processed to a 21-23 nt RNA species and the finding of Hamilton and Baulcombe that a 25 nt RNA correlates with post-transcriptional gene silencing in plants (Hamilton and Baulcombe, Science, 286:950-2 (1999)). Of the 16 cleavage sites we mapped (2 for dsRNA 'A,' 5 for dsRNA 'B,' and 9 for dsRNA 'C'), all but two reflect the 21-23 nt interval. One of the two exceptional cleavages was a weak cleavage site produced by dsRNA 'C' (indicated by an open blue circle in **FIG. 10**). This cleavage occurred 32 nt 5' to the next cleavage site. The other exception is particularly intriguing. After four cleavages spaced 21-23 nt apart, dsRNA 'C' caused cleavage of the mRNA just nine nt 3' to the previous cleavage site (red arrowhead in **FIG. 10**). This cleavage occurred in a run of seven uracil residues and appears to "reset" the ruler for cleavage; the next cleavage site was 21-23 nt 3' to the exceptional site. The three subsequent cleavage sites that we mapped were also spaced 21-23 nt apart. Curiously, of the sixteen cleavage sites caused by the three different dsRNAs, fourteen occur at uracil residues. The significance of this finding is not understood, but it suggests that mRNA cleavage is determined by a process which measures 21-23 nt intervals and which has a sequence preference for cleavage at uracil. Results show that the 21-23 nt RNA species produced by incubation of 500 bp dsRNA in the lysate caused sequence-specific interference in vitro when isolated from an acrylamide gel and added to a new RNAi reaction in place of the full-length dsRNA.

[0126] A Model for dsRNA-Directed mRNA Cleavage

[0127] Without wishing to be bound by theory, the biochemical data described herein, together with recent genetic experiments in *C. elegans* and Neurospora (Cogoni and Macino, Nature, 399:166-9 (1999); Grishok et al., Science, 287: 2494-7 (2000); Ketting et al., Cell, 99:133-41 (1999); Tabara et al., Cell, 99:123-32 (1999)), suggest a model for how dsRNA targets mRNA for destruction (**FIG. 11**). In this model, the dsRNA is first cleaved to 21-23 nt long fragments in a process likely to involve genes such as the *C. elegans* loci rde-1 and rde-4. The resulting fragments, probably as short asRNAs bound by RNAi-specific proteins, would then pair with the mRNA and recruit a nuclease that cleaves the mRNA. Alternatively, strand exchange could occur in a protein-RNA complex that transiently holds a 21-23 nt dsRNA fragment close to the mRNA. Separation of the two strands of the dsRNA following fragmentation might be assisted by an ATP-dependent RNA helicase, explaining the observed ATP enhancement of 21-23 nt RNA production.

[0128] It is likely that each small RNA fragment produces one, or at most two, cleavages in the mRNA, perhaps at the

5' or 3' ends of the 21-23 nt fragment. The small RNAs may be amplified by an RNA-directed RNA polymerase such as that encoded by the ego-1 gene in *C. elegans* (Smardon et al., Current Biology, 10:169-178 (2000)) or the qde-1 gene in Neurospora (Cogoni and Macino, Nature, 399:166-9 (1999)), producing long-lasting post-transcriptional gene silencing in the absence of the dsRNA that initiated the RNAi effect. Heritable RNAi in *C. elegans* requires the rde-1 and rde-4 genes to initiate, but not to persist in subsequent generations. The rde-2, rde-3, and mut-7 genes in *C. elegans* are required in the tissue where RNAi occurs, but are not required for initiation of heritable RNAi (Grishok et al., Science, in press 2000). These 'effector' genes (Grishok et al., Science, in press 2000) are likely to encode proteins functioning in the actual selection of mRNA targets and in their subsequent cleavage. ATP may be required at any of a number of steps during RNAi, including complex formation on the dsRNA, strand dissociation during or after dsRNA cleavage, pairing of the 21-23 nt RNAs with the target mRNA, mRNA cleavage, and recycling of the targeting complex. Testing these ideas with the in vitro RNAi system will be an important challenge for the future. Some genes involved in RNAi are also important for transposon silencing and co-suppresion. Co-suppression is a broad biological phenomenon spanning plants, insects and perhaps humans. The most likely mechanism in Drosophila melanogaster is transcriptional silencing (Pal-Bhanra et al, Cell 99: 35-36. Thus, 21-23 nt fragments are likely to be involved in transcriptional control, as well as in post-transcriptional cotrol.

Example 3

Isolated 21-23 mers caused Sequence-Specific Interference when Added to a New RNAi Reaction

[0129] Isolation of 21-23 nt Fragments from Incubation Reaction of 500 bp dsRNA in Lysate.

[0130] Double-stranded RNA (500 bp from) was incubated at 10 nM concentration in Drosophila embryo lysate for 3 h at 25° C. under standard conditions as described herein. After deproteinization of the sample, the 21-23 nt reaction products were separated from unprocessed dsRNA by denaturing polyacrylamide (15%) gel electrophoresis. For detection of the non-radiolabeled 21-23 nt fragments, an incubation reaction with radiolabeled dsRNA was loaded in a separate lane of the same gel. Gel slices containing the non-radioactive 21-23 nt fragments were cut out and the 21-23 nt fragments were eluted from the gel slices at 4° C. overnight in 0.4 ml 0.3 M NaCl. The RNA was recovered from the supernatant by ethanol precipitation and centrifugation. The RNA pellet was dissolved in 10 μl of lysis buffer. As control, gel slices slightly above and below the 21-23 nt band were also cut out and subjected to the same elution and precipitation procedures. Also, a non-incubated dsRNA loaded on the 15% gel and a gel slice corresponding to 21-23 nt fragments was cut out and eluted. All pellets from the control experiments were dissolved in 10 μl lysis buffer. The losses of RNA during recovery from gel slices by elution are approx. 50%.

[0131] Incubation of Purified 21-23 nt Fragments in a Translation-Based RNAi Assay

[0132] 1 μl of the eluted 21-23 mer or control RNA solution was used for a standard 10 μl RNAi incubation reaction (see above). The 21-23 mers were preincubated in the lysate containing reaction mixture for 10 or 30 min before the addition of the target and control mRNA. During pre-incubation, proteins involved in RNA interference may re-associate with the 21-23 mers due to a specific signal present on these RNAs. The incubation was continued for another hour to allow translation of the target and control mRNAs. The reaction was quenched by the addition of passive lysis buffer (Promega), and luciferase activity was measured. The RNA interference is the expressed as the ratio of target to control luciferase activity normalized by an RNA-free buffer control. Specific suppression of the target gene was observed with either 10 or 30 minutes preincubation. The suppression was reproducible and reduced the relative ratio of target to control by 2-3 fold. None of the RNA fragments isolated as controls showed specific interference. For comparison, incubation of 5 nM 500 bp dsRNA (10 min pre-incubation) affects the relative ratio of control to target gene approx. 30-fold.

[0133] Stability of Isolated 21-23 nt Fragments in a New Lysate Incubation Reaction.

[0134] Consistent with the observation of RNAi mediated by purified 21-23 nt RNA fragment, it was found that 35% of the input 21-23 nt RNA persists for more than 3 h in such an incubation reaction. This suggests that cellular factors associate with the deproteinized 21-23 nt fragments and reconstitute a functional mRNA-degrading particle. Signals connected with these 21-23 nt fragments, or their possible double stranded nature or specific lengths are likely responsible for this observation. The 21-23 nt fragments have a terminal 3' hydroxyl group, as evidenced by altered mobility on a sequencing gel following periodate treatment and beta-elimination.

Example 4

21-23-mers Purified by Non-Denaturing Methods Caused Sequence-Specific Interference when Added to a New RNAi Reaction.

[0135] Fifty nanomolar double-stranded RNA (501 bp Rr-luc dsRNA, as described in example 1) was incubated in a 1 ml in vitro reaction with lysate at 25° C. (see example 1). The reaction was then stopped by the addition of an equal volume of 2× PK buffer (see example 1) and proteinase K was added to a final concentration of 1.8 μg/μl. The reaction was incubated for an additional 1 h at 25° C., phenol extracted, and then the RNAs were precipitated with 3 volumes of ethanol. The ethanol precipitate was collected by centrifugation, and the pellet was resuspended in 100 μl of lysis buffer and applied to a Superdex HR 200 10/30 gel filtration column (Pharmacia) run in lysis buffer at 0.75 ml/min. 200 μl fractions were collected from the column. Twenty μl of 3 M sodium acetate and 20 μg glycogen was added to each fraction, and the RNA was recovered by precipitation with 3 volumes of ethanol. The precipitates were resuspended in 30 μl of lysis buffer. Column profiles following the fractionation of 32P-labeled input RNA are shown in **FIG. 13A.**

[0136] One microliter of each resuspended fraction was tested in a 10 μl standard in vitro RNAi reaction (see example 1). This procedure yields a concentration of RNA in the in vitro RNAi reaction that is approximately equal to

the concentration of that RNA species in the original reaction prior to loading on the column. The fractions were preincubated in the lysate containing reaction mixture for 30 min before the addition of 10 nM Rr-luc mRNA target and 10 nM Pp-luc control mRNA. During pre-incubation, proteins involved in RNA interference may re-associate with the 21-23-mers due to a specific signal present on these RNAs. The incubation was continued for another three hours to allow translation of the target and control mRNAs. The reaction was quenched by the addition of passive lysis buffer (Promega), and luciferase activity was measured. The suppression of Rr-luc mRNA target expression by the purified 21-23 nt fragments was reproducible and reduced the relative ratio of target to control by >30-fold, an amount comparable to a 50 nM 500 bp dsRNA control. Suppression of target mRNA expression was specific: little or no effect on the expression of the Pp-luc mRNA control was observed.

[0137] The data show that the both the fractions containing uncleaved dsRNA (fractions 3-5) or long, partially cleaved dsRNA (fractions 7-13) and the fractions containing the fully processed 21-23 nt siRNAs (fractions 41-50) mediate effective RNA interference in vitro **(FIG. 13B)**. Suppression of target mRNA expression was specific: little or no effect on the expression of the Pp-luc mRNA control was observed **(FIG. 13C)**. These data, together with those in the earlier examples, demonstrate that the 21-23 nt siRNAs are (1) true intermediates in the RNAi pathway and (2) effective mediators of RNA interference in vitro.

Example 5

21-Nucleotide siRNA Duplexes Mediate RNA Interference in Human Tissue Cultures

[0138] Methods

[0139] RNA Preparation

[0140] 21 nt RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified (Elbashir, S. M., Lendeckel, W. & Tuschl, T., *Genes & Dev.* 15, 188-200 (2001)), followed by Sep-Pak C18 cartridge (Waters, Milford, Mass., USA) purification (Tuschl, t., et al., *Biochemistry,* 32:11658-11668 (1993)). The siRNA sequences targeting GL2 (Acc. X65324) and GL3 luciferase (Acc. U47296) corresponded to the coding regions 153-173 relative to the first nucleotide of the start codon, siRNAs targeting RL (Acc. AF025846) corresponded to region 119-129 after the start codon. Longer RNAs were transcribed with T7 RNA polymerase from PCR products, followed by gel and Sep-Pak purification. The 49 and 484 bp GL2 or GL3 dsRNAs corresponded to position 113-161 and 113-596, respectively, relative to the start of translation; the 50 and 501 bp RL dsRNAs corresponded to position 118-167 and 118-618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (Kehlenbach, R. H., et al., *J. Cell Biol.,* 141:863-874 (1998)), whereby 50 and 501 bp hG dsRNA corresponded to position 118-167 and 118-618, respectively, to the start codon.

[0141] For annealing of siRNAs, 20 μM single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90° C. followed by 1 h at 37° C. The 37° C. incubation step was extended overnight for the 50 and 500 bp dsRNAs, and these annealing reactions were performed at 8.4 μM and 0.84 μM strand concentrations, respectively.

[0142] Cell Culture

[0143] S2 cells were propagated in Schneider's Drosophila medium (Life Technologies) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 25° C. 293, NIH/3T3, HeLa S3, COS-7 cells were grown at 37° C. in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were regularly passaged to maintain exponential growth. 24 h before transfection at approx. 80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics ($1\text{-}3\times10^5$ cells/ml) and transferred to 24-well plates (500 μl/well). S2 cells were not trypsinized before splitting. Transfection was carried out with Lipofectamine 2000 reagent (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 μg pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 μg pRL-TK (Promega), and 0.28 μg siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 μl per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cell lines after co-transfection of 1.1 μg hGFP-encoding pAD3[22] and 0.28 μg invGL2 siRNA, and were 70-90%. Reporter plasmids were amplified in XL-1 Blue (Strategene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

[0144] Results

[0145] RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) homologous in sequence to the silenced gene (Fire, A., *Trends Genet.,* 15:358-363 (1999); Sharp, P.A. & Zamore, P. D., *Science,* 287:2431-2433 (2000); Sijen, T. & Kooter, J. M., *Bioessays,* 22:520-531 (2000); Bass, B. L., *Cell,* 101:235-238 (2000); Hammond, S. M., et al., *Nat. Rev. Genet.,* 2:110-119 (2001)). The mediators of sequence-specific mRNA degradation are 21 and 22 nt small interfering RNAs (siRNAs) generated by RNase III cleavage from longer dsRNAs[6-10] (Hamilton, A. J. &Baulcombe, D. C, *Science,* 286:950-952 (1999); Hammond, S. M., et al., Nature, 404:293-296 (2000); Zamore, P. D., et al., *Cell,* 101:25-33 (2000); Bernstein, E., et al, *Naature,* 409:363-366 (2001); Elbashir, S. M., et al., *Genes & Dev.,* 15:188-200 (2001)). As shown herein, 21 nt siRNA duplexes are able to specifically suppress reporter gene expression in multiple mammalian tissue cultures, including human embryonic kidney (293) and HeLa cells. In contrast to 50 or 500 bp dsRNAs, siRNAs do not activate the interferon response. These results indicate that siRNA duplexes are a general tool for sequence-specific inactivation of gene function in mammalian cells.

[0146] Base-paired 21 and 22 nt siRNAs with overhanging 3' ends mediate efficient sequence-specific mRNA degradation in lysates prepared from *D. melanogaster* embryos (Elbashir, S. M., et al., *Genes & Dev.,* 15:188-200 (2001)). To test whether siRNAs are also capable of mediating RNAi

in tissue culture, 21 nt siRNA duplexes with symmetric 2 nt 3' overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases (**FIGS. 14A, 14B**) were constructed. The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL, into *D. melanogaster* Schneider S2 cells or mammalian cells using cationic liposomes. Luciferase activities were determined 20 h after transfection. In all cell lines tested, specific reduction of the expression of the reporter genes in the presence of cognate siRNA duplexes was observed (FIGS. **15A-15J**). Remarkably, the absolute luciferase expression levels were unaffected by non-cognate siRNAs, indicating the absence of harmful side effects by 21 nt RNA duplexes (e.g. FIGS. **16A-16**D, for HeLa cells). In *D. melanogaster* S2 cells (**FIGS. 15A, 15B**), the specific inhibition of luciferases was complete, and similar to results previously obtained for longer dsRNAs (Hammond, S. M., et al., *Nature,* 404:293-296 (2000); Caplen, N. J., et al., sGene, 252:95-105 (2000); Clemens, M & Williams, B., *Cell,* 13:565-572 (1978); Ui-Tei, K., et al., *FEBS Letters,* 479:79-82 (2000)). In mammalian cells, where the reporter genes were 50- to 100-fold stronger expressed, the specific suppression was less complete (FIGS. **15C-15J**). GL2 expression was reduced 3- to 12-fold, GL3 expression 9- to 25-fold, and RL expression 1- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (**FIGS. 15I, 15J**). It is likely that the lack of reduction of RL expression in 293 cells is due to its 5- to 20-fold higher expression compared to any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

[0147] The 2 nt 3' overhang in all siRNA duplexes, except for uGL2, was composed of (2'-deoxy) thymidine. Substitution of uridine by thymidine in the 3' overhang was well tolerated in the *D. melanogaster* in vitro system, and the sequence of the overhang was uncritical for target recognition (Elbashir, S. M., et al., *Genes & Dev.,* 15:188-200 (2001)). The thymidine overhang was chosen, because it is supposed to enhance nuclease resistance of siRNAs in the tissue culture medium and within transfected cells. Indeed, the thymidine-modified GL2 siRNA was slightly more potent than the unmodified uGL2 siRNA in all cell lines tested (**FIGS. 15A, 15C, 15**E, **15**G, **15I**). It is conceivable that further modifications of the 3' overhanging nucleotides will provide additional benefits to the delivery and stability of siRNA duplexes.

[0148] In co-transfection experiments, 25 nM siRNA duplexes with respect to the final volume of tissue culture medium were used (FIGS. **15A-15J, 16A-16F**). Increasing the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies due to competition for liposome encapsulation between plasmid DNA and siRNA. Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect, even though the siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing, and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene targeting experiments.

[0149] In order to monitor the effect of longer dsRNAs on mammalian cells, 50 and 500 bp dsRNAs cognate to the reporter genes were prepared. As non-specific control, dsRNAs from humanized GFP (hG) (Kehlenbach, R. H., et al., *J. Cell Biol.,* 141:863874 (1998)) was used. When dsRNAs were co-transfected, in identical amounts (not concentrations) to the siRNA duplexes, the reporter gene expression was strongly and unspecifically reduced. This effect is illustrated for HeLa cells as a representative example (FIGS. **16A-16**D). The absolute luciferase activities were decreased unspecifically 10- to 20-fold by 50 bp dsRNA, and 20- to 200-fold by 500 bp dsRNA co-transfection, respectively. Similar unspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold unspecific reduction was observed only for 500 bp dsRNAs. Unspecific reduction in reporter gene expression by dsRNA >30 bp was expected as part of the interferon response (Matthews, M., Interactions between viruses and the cellular machinery for protein synthesis in *Translational Control* (eds., Hershey, J., Matthews, M. & Sonenberg, N.) 505-548 (Cold Spring Harbor Laboratory Press, Plainview, N.Y.; 1996); Kumar, M. & Carmichael, G. G., *Microbiol. Mol. Biol. Rev.,* 62:1415-1434 (1998); Stark, G. R., et al., *Annu. Rev. Biochem.,* 67:227-264 (1998)). Surprisingly, despite the strong unspecific decrease in reporter gene expression, additional sequence-specific, dsRNA-mediated silencing were reproducibly detected. The specific silencing effects, however, were only apparent when the relative reporter gene activities were normalized to the hG dsRNA controls (**FIGS. 16E, 16F**). A 2- to 10-fold specific reduction in response to cognate dsRNA was observed, also in the other three mammalian cell lines tested. Specific silencing effects with dsRNAs (356-1662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments (Ui-Tei, K., et al., *FEBS Letters,* 479:79-82 (2000)). Also, CHO-K1 cells appear to be deficient in the interferon response. In another report, 293, NIH/3T3, and BHK-21 cells were tested for RNAi using luciferase/lacZ reporter combinations and 829 bp specific lacZ or 717 bp unspecific GFP dsRNA (Caplen, N. J., et al., *Gene,* 252:95105 (2000)). The failure of detecting RNAi in this case is likely due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, the results described herein indicate that RNAi is active in mammalian cells, but that the silencing effect is difficult to detect if the interferon system is activated by dsRNA >30 bp.

[0150] The mechanism of the 21 nt siRNA-mediated interference process in mammalian cells remains to be uncovered, and silencing may occur post-transcriptional and/or transcriptional. In *D. melanogaster* lysate, siRNA duplexes mediate post-transcriptional gene silencing by reconstitution of a siRNA-protein complexes (siRNPs), which are guiding mRNA recognition and targeted cleavage (Hammond, S. M., et al., *Nature,* 404:293-296 (2000); Zamore, P. D., et al., *Cell,* 101:25-33 (2000); Elbashir, S. M., et al., *Genes & Dev.,* 15:188-200 (2001)). In plants, dsRNA-mediated post-transcriptional silencing has also been linked to RNA-directed DNA methylation, which may also be directed by 21 nt siRNAs (Wassenegger, M., *Plant Mol. Biol,* 43:203-220

(2000); Finnegan, E. J., et al., *Curr. Biol,* 11:R99-R102 (2000)). Methylation of promoter regions can lead to transcriptional silencing (Metter, M. F., et al., *EMBO J.,* 19:5194-5201 (2000)), but methylation in coding sequences must not (Wang, M. -B., *RNA,* 7:16-28 (2001)). DNA methylation and transcriptional silencing in mammals are well-documented processes (Kass, S. U., et al., *Trends Genet.,* 13:444-449 (1997); Razin, A., *EMBO J,* 17:4905-4908 (1998)), yet they have not been linked to post-transcriptional silencing. Methylation in mammals is predominantly directed towards CpG residues. Because there is no CpG in the RL siRNA, but RL siRNA mediates specific silencing in mammalian tissue culture, it is unlikely that DNA methylation is critical for our observed silencing process. In summary, described herein, is siRNA-mediated gene silencing in mammalian cells. The use of 21 nt siRNAs holds great promise for inactivation of gene function in human tissue culture and the development of gene-specific therapeutics.

[0151] While this invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims

What is claimed is:

1. Isolated RNA of from about 21 to about 23 nucleotides that mediates RNA interference of an mRNA to which it corresponds.

2. Isolated RNA of claim 1 that comprises a terminal 3' hydroxyl group.

3. Isolated RNA of claim 1 which is chemically synthesized RNA or an analog of a naturally occurring RNA.

4. An analog of isolated RNA of claim 1, wherein the analog differs from the RNA of claim 1 by the addition, deletion, substitution or alteration of one or more nucleotides.

5. Isolated RNA of from about 21 to about 23 nucleotides that inactivates a corresponding gene by transcriptional silencing.

6. A soluble extract that mediates RNA interference.

7. The soluble extract of claim 6, wherein the extract is derived from Drosophila embryos.

8. The soluble extract of claim 7 wherein the extract is derived from syncytial blastoderm Drosophila embryos.

9. A method of producing RNA of from about 21 to about 23 nucleotides in length comprising:

(a) combining double-stranded RNA with a soluble extract that mediates RNA interference, thereby producing a combination; and

(b) maintaining the combination of a) under conditions in which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides in length.

10. The method of claim 9, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

11. The method of claim 9 further comprising isolating the RNA of from about 21 to about 23 nucleotides from the combination.

12. RNA of about 21 to about 23 nucleotides produced by the method of claim 9.

13. A method of producing RNA of from about 21 to about 23 nucleotides in length that mediates RNA interference of mRNA of a gene to be degraded, comprising:

(a) combining double-stranded RNA that corresponds to a sequence of the gene to be degraded with a soluble extract that mediates RNA interference, thereby producing a combination; and

(b) maintaining the combination of (a) under conditions under which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides that mediates RNA interference of the mRNA of the gene to be degraded, thereby producing RNA of from about 21 to about 23 nucleotides that mediates RNA interference of the mRNA.

14. The method of claim 13, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

15. The method of claim 13 further comprising isolating RNA of from about 21 to about 23 nucleotides from the combination.

16. Isolated RNA of from about 21 to about 23 nucleotides produced by the method of claim 15.

17. A method of mediating RNA interference of mRNA of a gene in a cell or organism comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into the cell or organism;

(b) maintaining the cell or organism produced in (a) under conditions under which degradation of the mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism.

18. The method of claim 17 wherein the RNA of (a) is a chemically synthesized mRNA or an analog of naturally occurring RNA.

19. The method of claim 17, wherein the gene encodes a cellular mRNA or a viral mRNA.

20. A method of mediating RNA interference of mRNA of a gene in a cell or organism in which RNA interference occurs, comprising:

(a) combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract that mediates RNA interference, thereby producing a combination;

(b) maintaining the combination produced in (a) under conditions under which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides, thereby producing RNA of from about 21 to about 23 nucleotides;

(c) isolating RNA of from about 21 to about 23 nucleotides produced in (b);

(d) introducing RNA isolated in (c) into the cell or organism; and

(e) maintaining the cell or organism produced in (d) under conditions under which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism.

21. The method of claim 20, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

22. The method of claim 20, wherein the RNA is isolated using gel electrophoresis.

23. A method of mediating RNA interference of mRNA of a gene in a cell or organism in which RNA interference occurs, comprising: (a) introducing into the cell or organism RNA of from about 21 to about 23 nucleotides that mediates RNA interference of mRNA of the gene, thereby producing a cell or organism that contains the RNA and (b) maintaining the cell or organism that contains the RNA under conditions under which RNA interference occurs, thereby mediating RNA interference of mRNA of the gene in the cell or organism.

24. The method of claim 23, wherein the RNA of from about 21 to about 23 nucleotides is chemically synthesized RNA or an analog of RNA that mediates RNA interference.

25. The method of claim 23, wherein the gene encodes a cellular mRNA or a viral mRNA.

26. A knockdown cell or organism generated by the method of claim 23.

27. The knockdown cell or organism of claim 26, wherein the cell or organism mimics a disease.

28. A method of examining the function of a gene in a cell or organism comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides that targets mRNA of the gene for degradation into the cell or organism, thereby producing a test cell or test organism;

(b) maintaining the test cell or test organism under conditions under which degradation of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is degraded; and

(c) observing the phenotype of the test cell or test organism produced in (b) and, optionally, comparing the phenotype observed to that of an appropriate control cell or control organism, thereby providing information about the function of the gene.

29. The method of claim 28 wherein the RNA introduced in (a) is chemically synthesized or an analog of RNA that mediates RNA interference.

30. A method of examining the function of a gene in a cell or organism comprising

(a) combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract that mediates RNA interference, thereby producing a combination;

(b) maintaining the combination produced in (a) under conditions under which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides, whereby RNA of about 21 to about 23 nucleotides is produced;

(c) isolating RNA of about 21 to about 23 nucleotides produced in (b);

(d) introducing the RNA isolated in (c) into the cell or organism, thereby producing a test cell or test organism;

(e) maintaining the test cell or test organism under conditions under which degradation of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is degraded; and

(f) observing the phenotype of the test cell or test organism produced in (e) and, optionally, comparing the phenotype observed to that of an appropriate control, thereby providing information about the function of the gene.

31. The method of claim 30, wherein the RNA comprises a terminal 3' hydroxyl group.

32. The method of claim 30, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

33. The method of claim 30, wherein the RNA is isolated using gel electrophoresis.

34. A composition comprising biochemical components of a Drosophila cell that process dsRNA to RNA of about 21 to about 23 nucleotides and a suitable carrier.

35. A composition comprising biochemical components of a cell that target mRNA of a gene to be degraded by RNA of about 21 to about 23 nucleotides.

36. A method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNA of from about 21 to about 23 nucleotides that targets the mRNA of the protein for degradation.

37. The method of claim 36 wherein RNA of from about 21 to about 23 nucleotides is chemically synthesized or an analog of RNA that mediates RNA interference.

38. A method of assessing whether an agent acts on a gene product comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into a cell or organism;

(b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs,

(c) introducing the agent into the cell or organism of (b); and

(d) determining whether the agent has an effect on the cell or organism, wherein if the agent has no effect on the cell or organism then the agent acts on the gene product or on a biological pathway that involves the gene product.

39. The method of claim 38, wherein the RNA of from about 21 to about 23 nucleotides is chemically synthesized or an analog of RNA that mediates RNA interference.

40. A method of assessing whether a gene product is a suitable target for drug discovery comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into a cell or organism;

(b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs resulting in decreased expression of the gene; and

(c) determining the effect of the decreased expression of the gene on the cell or organism, wherein if decreased expression has an effect, then the gene product is a target for drug discovery.

41. The method of claim 40, wherein the RNA of from about 21 to about 23 nucleotides is synthetic RNA or an analog of RNA that mediates RNA interference.

42. A gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

43. A pharmaceutical composition comprising RNA of from about 21 to about 23 nucleotides that mediates RNA interference and an appropriate carrier.

44. A method of producing knockdown cells, comprising introducing into cells in which a gene is to be knocked down RNA of about 21 to about 23 nt that targets the mRNA corresponding to the gene and maintaining the resulting cells under conditions under which RNAi occurs, resulting in degradation of the mRNA of the gene, thereby producing knockdown cells.

45. The method of claim 44, wherein the RNA of about 21 to about 23 nucleotides is synthetic RNA or an analog of RNA that mediates RNA interference.

46. A method of identifying target sites within mRNA that are efficiently cleaved by the RNAi process, comprising combining dsRNA corresponding to a sequence of a gene to be degraded, labeled mRNA corresponding to the gene and a soluble extract that mediates RNA interference, thereby producing a combination; maintaining the combination under conditions under which the dsRNA is degraded and identifying sites in the mRNA that are efficiently cleaved.

47. A method of identifying 21-23 nt RNAs that efficiently mediate RNAi, wherein said 21-23 nt RNAs span the target sites identified within the mRNA by the method of claim 46.

48. RNA of claim 16, isolated using gel electrophoresis.

49. RNA of claim 16, isolated using non-denaturing methods.

50. RNA of claim 16, isolated using non-denaturing column chromatography.

* * * * *

EXHIBIT 7

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Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

00126325.0

Der Präsident des Europäischen Patentamts;
Im Auftrag

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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RNA interference mediating small RNA molecules

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RNA Interference Mediating Small RNA molecules

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Description

The present invention relates to sequence and structural features of double-stranded (ds)RNA molecules required to mediate target-specific nucleic acid modifications such as RNA-interference and/or DNA methylation.

The term "RNA interference" (RNAi) was coined after the discovery that injection of dsRNA into the nematode C. elegans leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA (Fire et al., 1998). RNAi was subsequently also observed in insects, frogs (Oelgeschlager et al., 2000), and other animals including mice (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000) and is likely to also exist in human. RNAi is closely linked to the post-transcriptional gene-silencing (PTGS) mechanism of co-suppression in plants and quelling in fungi (Catalanotto et al., 2000; Cogoni and Macino, 1999; Dalmay et al., 2000; Ketting and Plasterk, 2000; Mourrain et al., 2000; Smardon et al., 2000) and some components of the RNAi machinery are also necessary for post-transcriptional silencing by co-suppression (Catalanotto et al., 2000; Dernburg et al., 2000; Ketting and Plasterk, 2000). The topic has also been reviewed recently (Bass, 2000; Bosher and Labouesse, 2000; Fire, 1999; Plasterk and Ketting, 2000; Sharp, 1999; Sijen and Kooter, 2000), see also the entire issue of Plant Molecular Biology, vol. 43, issue 2/3, (2000).

In plants, in addition to PTGS, introduced transgenes can also lead to transcriptional gene silencing via RNA-directed DNA methylation of cytosines (see references in Wassenegger, 2000). Genomic targets as short as 30 bp are methylated in plants in an RNA-directed manner (Pelissier, 2000). DNA methylation is also present in mammals.

The natural function of RNAi and co-suppression appears to be protection of the genome against invasion by mobile genetic elements such as retro-transposons and viruses which produce aberrant RNA or dsRNA in the host cell when they become active (Jensen et al, 1999; Ketting et al., 1999; Ratcliff et al., 1999; Tabara et al., 1999). Specific mRNA degradation prevents transposon and virus replication although some viruses are able to overcome or prevent this process by expressing proteins that suppress PTGS (Lucy et al., 2000; Voinnet et al., 2000).

DsRNA triggers the specific degradation of homologous RNAs only within the region of identity with the dsRNA (Zamore et al., 2000). The dsRNA is processed to 21-23 nt RNA fragments and the target RNA cleavage sites are regularly spaced 21-23 nt apart. It has therefore been suggested that the 21-23 nt fragments are the guide RNAs for target recognition (Zamore et al., 2000). These short RNAs were also detected in extracts prepared from D. melanogaster Schneider 2 cells which were transfected with dsRNA prior to cell lysis (Hammond et al., 2000), however, the fractions that displayed sequence-specific nuclease activity also contained a large fraction of residual dsRNA. The role of the 21-23 nt fragments in guiding mRNA cleavage is further supported by the observation that 21-23 nt fragments isolated from processed dsRNA are able, to some extent, to mediate specific mRNA degradation (Zamore et al., 2000). RNA molecules of similar size also accumulate in plant tissue that exhibits PTGS (Hamilton and Baulcombe, 1999).

Here, we use the established Drosophila in vitro system (Tuschl et al., 1999; Zamore et al., 2000) to further explore the mechanism of RNAi. We demonstrate that short 21 and 22 nt RNAs, when base-paired with 3' overhanging ends, act as the guide RNAs for sequence-specific mRNA degradation. Short 30 bp dsRNAs are unable to mediate RNAi because they are no longer processed to 21 and 22 nt RNAs. Furthermore, we defined the target RNA cleavage sites relative to the 21 and 22 nt short

interfering RNAs (siRNAs) and provide evidence that the direction of dsRNA processing determines whether a sense or an antisense target RNA can be cleaved by the produced siRNP endonuclease complex. Further, the siRNAs may also be important tools for transcriptional modulating, e.g. silencing of mammalian genes by guiding DNA methylation.

The object underlying the present invention is to provide novel agents capable of mediating target-specific RNA interference or other target-specific nucleic acid modifications such as DNA methylation, said agents having an improved efficacy and safety compared to prior art agents.

The solution of this problem is provided by an isolated double-stranded RNA molecule, wherein each RNA strand has a length from 19-23 nucleotides, wherein said RNA molecule is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation. Preferably at least one strand has a 3'-overhang from 1-5 nucleotides. The other strand may be blunt-ended or has up to 6 nucleotides 3' overhang. Also, if both strands of the dsRNA are exactly 21 or 22 nt, it is possible to observe some RNA interference when both ends are blunt (0 nt overhang). The RNA molecule is preferably a synthetic RNA molecule which is substantially free from contaminants occurring in cell extracts, e.g. from Drosophila embryos. Further, the RNA molecule is preferably substantially free from any non-target-specific contaminants, particularly non-target-specific RNA molecules e.g. from contaminants occuring in cell extracts.

Surprisingly, it was found that synthetic short double-stranded RNA molecules particularly with overhanging 3'-ends are sequence-specific mediators of RNAi and mediate efficient target-RNA cleavage, wherein the cleavage site is located near the center of the region spanned by the guiding short RNA.

Preferably, each strand of the RNA molecule has a length from 20-22 nucleotides, wherein the length of each strand may be the same or different. Preferably, the length of the 3'-overhang reaches from 1-3 nucleotides, wherein the length of the overhang may be the same or different for each strand. The RNA-strands preferably have 3'-hydroxyl groups. The 5'-terminus preferably comprises a phosphate, diphosphate, triphosphate or hydroxyl group. The most effective dsRNAs are composed of two 21 nt strands which are paired such that 1-3, particularly 2 nt 3' overhangs are present on both ends of the dsRNA. Pyrimidine nucleotides in the 3' overhang are more effective in mediating RNA interference than purine nucleotides. Also, the nucleotides present in the 3' overhang do not seem to contribute to the specificity of target RNA recognition.

Surprisingly, the double-stranded RNA molecules of the present invention exhibit a high in vivo stability in serum or in growth medium for cell cultures. In order to further enhance the stability, the 3'-overhangs may be stablized against degradation, e.g. they may be selected such that they consist of purine nucleotides, particularly adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g. Substitution of uridine 2 nt 3' overhangs by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNA interference. The absence of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium.

In an especially preferred embodiment of the present invention the RNA molecule may contain at least one modified nucleotide analogue. The nucleotide analogues may be located at positions where the target-specific activity, e.g. the RNAi mediating activity is not substantially effected, e.g. in a region at the 5'-end and/or the 3'-end of the double-stranded RNA molecule. Particularly, the overhangs may be stabilized by incorporating modified nucleotide analogues.

Preferred nucleotide analogues are selected from sugar- or backbone-modified ribonucleotides. It should be noted, however, that also nucleobase-modified ribonucleotides, i.e. ribonucleotides, containing a non-naturally occurring nucleobase instead of a naturally occurring nucleobase such as uridines or cytidines modified at the 5-position, e.g. 5-(2-amino)propyl uridine, 5-bromo uridine; adenosines and guanosines modified at the 8-position, e.g. 8-bromo guanosine; deaza nucleotides, e.g. 7-deaza-adenosine; O- and N-alkylated nucleotides, e.g. N6-methyl adenosine are suitable. In preferred sugar-modified ribonucleotides the 2´ OH-group is replaced by a group selected from H, OR, R, halo, SH, SR, NH_2, NHR, NR_2 or CN, wherein R is C_1-C_6 alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I. In preferred backbone-modified ribonucleotides the phosphoester group connecting to adjacent ribonucleotides is replaced by a modified group, e.g. of phosphothioate group. It should be noted that the above modifications may be combined.

The sequence of the double-stranded RNA molecule of the present invention has to have a sufficient identity to a nucleic acid target molecule in order to mediate target-specific RNAi and/or DNA methylation. Preferably, the sequence has an identity of at least 50%, particularly of at least 70% to the desired target molecule in the double-stranded portion of the RNA molecule. More preferably, the identity is at least 70% and most preferably 100% in the double-stranded portion of the RNA molecule. The identity of a double-stranded RNA molecule to a predetermined nucleic acid target molecule, e.g. an mRNA target molecule may be determined as follows:

$$I = \frac{n}{L} \times 100$$

wherein I is the identity in percent, n is the number of identical nucleotides in the double-stranded portion of the ds RNA and the target and L is the length of the sequence overlap of the double-stranded portion of the dsRNA and the target.

The double-stranded RNA molecule of the invention may be prepared by a method comprising the steps:

(a) synthesizing two RNA strands each having a length from 19-23 nucleotides, wherein said RNA strands are capable of forming a double-stranded RNA molecule, wherein preferably at least one strand has a 3'-overhang from 1-5 nucleotides,

(b) combining the synthesized RNA strands under conditions, wherein a double-stranded RNA molecule is formed, which is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation.

Methods of synthesizing RNA molecules are known in the art. In this context, it is particularly referred to chemical synthesis methods as described in Verma and Eckstein (1998).

The single-stranded RNAs can also be prepared by enzymatic transcription from synthetic DNA templates or from DNA plasmids isolated from recombinant bacteria. Typically, phage RNA polymerases are used such as T7, T3 or SP6 RNA polymerase (Milligan and Uhlenbeck (1989)).

A further aspect of the present invention relates to a method of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation in a cell or an organism comprising the steps:

(a) contacting the cell or organism with the double-stranded RNA molecule of the invention and

(b) mediating a target-specific nucleic acid modificiation effected by the double-stranded RNA towards a target nucleic acid having a

sequence portion substantially corresponding to the double-stranded RNA.

The method of the invention may be used for determining the function of a gene in a cell or an organism or even for modulating the function of a gene in a cell or an organism, being capable of mediating RNA interference. The cell is preferably a eukaryotic cell or a cell line, e.g. a plant cell or an animal cell, such as a mammalian cell, e.g. an embryonic cell, a pluripotent stem cell, a tumor cell, e.g. a teratocarcinoma cell or a virus-infected cell. The organism is preferably a eukaryotic organism, e.g. a plant or an animal, such as a mammal, particularly a human.

The target gene to which the RNA molecule of the invention is directed may be associated with a pathological condition. For example, the gene may be a pathogen-associated gene, e.g. a viral gene, a tumor-associated gene or an autoimmune disease-associated gene. The target gene may also be a heterologous gene expressed in a recombinant cell or a genetically altered organism. By determinating or modulating, particularly, inhibiting the function of such a gene valuable information and therapeutic benefits in the agricultural field or in the medicine or veterinary medicine field may be obtained.

The dsRNA is usually administered as a pharmaceutical composition. The administration may be carried out by known methods, wherein a nucleic acid is introduced into a desired target cell in vitro or in vivo. Commonly used gene transfer techniques include calcium phosphate, DEAE-dextran, electroporation and microinjection and viral methods (Graham, F.L. and van der Eb, A.J. (1973) Virol. 52, 456; McCutchan, J.H. and Pagano, J.S. (1968), J. Natl. Cancer Inst. 41, 351; Chu, G. et al (1987), Nucl. Acids Res. 15, 1311; Fraley, R. et al. (1980), J. Biol. Chem. 255, 10431; Capecchi, M.R. (1980), Cell 22, 479). A recent addition to this arsenal of techniques for the introduction of DNA into cells is the use of cationic liposomes

(Felgner, P.L. et al. (1987), Proc. Natl. Acad. Sci USA 84, 7413). Commercially available cationic lipid formulations are e.g. Tfx 50 (Promega) or Lipofectamin2000 (Life Technologies).

Thus, the invention also relates to a pharmaceutical composition containing as an active agent at least one double-stranded RNA molecule as described above and a pharmaceutical carrier. The composition may be used for diagnostic and for therapeutic applications.

For diagnostic or therapeutic applications, the composition may be in form of a solution, e.g. an injectable solution, a cream, ointment, tablet, suspension or the like. The composition may be administered in any suitable way, e.g. by injection, by oral, topical, nasal, rectal application etc. The carrier may be any suitable pharmaceutical carrier. Preferably, a carrier is used, which is capable of increasing the efficacy of the RNA molecules to enter the target-cells. Suitable examples of such carriers are liposomes, particularly cationic liposomes.

Further, the present invention is explained in more detail in the following figures and examples.

Figure Legends

Figure 1: Double-stranded RNA as short as 38 bp can mediate RNAi. (A) Graphic representation of dsRNAs used for targeting Pp-luc mRNA. Three series of blunt-ended dsRNAs covering a range of 29 to 504 bp were prepared. The position of the first nucleotide of the sense strand of the dsRNA is indicated relative to the start codon of Pp-luc mRNA (p1). (B) RNA interference assay (Tuschl et al., 1999) . Ratios of target Pp-luc to control Rr-luc activity were normalized to a buffer control (black bar). DsRNAs (5 nM) were preincubated in Drosophila lysate for 15 min at 25°C prior to the addition of 7-methyl-guanosine-capped Pp-luc and Rr-luc

mRNAs (~50 pM). The incubation was continued for another hour and then analyzed by the dual luciferase assay (Promega). The data are the average from at least four independent experiments ± standard deviation.

Figure 2: A 29 bp dsRNA is no longer processed to 21-23 nt fragments. Time course of 21-23 mer formation from processing of internally ^{32}P-labeled dsRNAs (5 nM) in the Drosophila lysate. The length and source of the dsRNA are indicated. An RNA size marker (M) has been loaded in the left lane and the fragment sizes are indicated. Double bands at time zero are due to incompletely denatured dsRNA.

Figure 3: Short dsRNAs cleave the mRNA target only once.
(A) Denaturing gel electrophoreses of the stable 5′ cleavage products produced by 1 h incubation of 10 nM sense or antisense RNA ^{32}P-labeled at the cap with 10 nM dsRNAs of the p133 series in Drosophila lysate. Length markers were generated by partial nuclease T1 digestion and partial alkaline hydrolysis (OH) of the cap-labeled target RNA. The regions targeted by the dsRNAs are indicated as black bars on both sides. The 20-23 nt spacing between the predominant cleavage sites for the 111 bp long dsRNA is shown. The horizontal arrow indicates unspecific cleavage not due to RNAi. (B) Position of the cleavage sites on sense and antisense target RNAs. The sequences of the capped 177 nt sense and 180 nt antisense target RNAs are represented in antiparallel orientation such that complementary sequence are opposing each other. The region targeted by the different dsRNAs are indicated by differently colored bars positioned between sense and antisense target sequences. Cleavage sites are indicated by circles: large circle for strong cleavage, small circle for weak cleavage. The ^{32}P-radiolabeled phosphate group is marked by an asterisk.

Figure 4: 21 and 22 nt RNA fragments are generated by an RNase III-like mechanism.

(A) Sequences of ~21 nt RNAs after dsRNA processing. The ~21 nt RNA fragments generated by dsRNA processing were directionally cloned and sequenced. Oligoribonucleotides originating from the sense strand of the dsRNA are indicated as blue lines, those originating from the antisense strand as red lines. Thick bars are used if the same sequence was present in multiple clones, the number at the right indicating the frequency. The target RNA cleavage sites mediated by the dsRNA are indicated as orange circles, large circle for strong cleavage, small circle for weak cleavage (see Figure 3B). Circles on top of the sense strand indicated cleavage sites within the sense target and circles at the bottom of the dsRNA indicate cleavage site in the antisense target. Up to five additional nucleotides were identified in ~21 nt fragments derived from the 3' ends of the dsRNA. These nucleotides are random combinations of predominantly C, G, or A residues and were most likely added in an untemplated fashion during T7 transcription of the dsRNA-constituting strands. (B) Two-dimensional TLC analysis of the nucleotide composition of ~21 nt RNAs. The ~21 nt RNAs were generated by incubation of internally radiolabeled 504 bp Pp-luc dsRNA in Drosophila lysate, gel-purified, and then digested to mononucleotides with nuclease P1 (top row) or ribonuclease T2 (bottom row). The dsRNA was internally radiolabeled by transcription in the presence of one of the indicated α-^{32}P nucleoside triphosphates. Radioactivity was detected by phosphorimaging. Nucleoside 5'-monophosphates, nucleoside 3'-monophosphates, nucleoside 5',3'-diphosphates, and inorganic phosphate are indicated as pN, Np, pNp, and p_i, respectively. Black circles indicate UV-absorbing spots from non-radioactive carrier nucleotides. The 3',5'-bisphosphates (red circles) were identified by co-migration with radiolabeled standards prepared by 5'-phosphorylation of nucleoside 3'-monophosphates with T4 polynucleotide kinase and γ-^{32}P-ATP.

Figure 5: Synthetic 21 and 22 nt RNAs Mediate Target RNA Cleavage (A) Graphic representation of control 52 bp dsRNA and synthetic 21 and 22 nt dsRNAs. The sense strand of 21 and 22 nt short interfering RNAs (siRNAs) is shown blue, the antisense strand in red. The sequences of the siRNAs were derived from the cloned fragments of 52 and 111 bp dsRNAs (Figure 4A), except for the 22 nt antisense strand of duplex 5. The siRNAs in duplex 6 and 7 were unique to the 111 bp dsRNA processing reaction. The two 3′ overhanging nucleotides indicated in green are present in the sequence of the synthetic antisense strand of duplexes 1 and 3. Both strands of the control 52 bp dsRNA were prepared by in vitro transcription and a fraction of transcripts may contain untemplated 3′ nucleotide addition. The target RNA cleavage sites directed by the siRNA duplexes are indicated as orange circles (see legend to Figure 4A) and were determined as shown in Figure 5B. (B) Position of the cleavage sites on sense and antisense target RNAs. The target RNA sequences are as described in Figure 3B. Control 52 bp dsRNA (10 nM) or 21 and 22 nt RNA duplexes 1-7 (100 nM) were incubated with target RNA for 2.5 h at 25°C in Drosophila lysate. The stable 5′ cleavage products were resolved on the gel. The cleavage sites are indicated in Figure 5A. The region targeted by the 52 bp dsRNA or the sense (s) or antisense (as) strands are indicated by the black bars to the side of the gel. The cleavage sites are all located within the region of identity of the dsRNAs. For precise determination of the cleavage sites of the antisense strand, a lower percentage gel was used.

Figure 6: Long 3′ overhangs on short dsRNAs inhibit RNAi (A) Graphic representation of 52 bp dsRNA constructs. The 3′ extensions of sense and antisense strand are indicated in blue and red, respectively. The observed cleavage sites on the target RNAs are represented as orange circles analogous to Figure 4A and were determined as shown in Figure 6B. (B) Position of the cleavage sites on sense and antisense target RNAs. The target RNA sequences are as described in Figure 3B. DsRNA (10 nM) was incubated with target RNA for 2.5 h at 25°C in Drosophila lysate. The

stable 5′ cleavage products were resolved on the gel. The major cleavage sites are indicated with a horizontal arrow and also represented in Figure 6A. The region targeted by the 52 bp dsRNA is represented as black bar at both sides of the gel.

Figure 7: Proposed Model for RNAi

RNAi is predicted to begin with processing of dsRNA (sense strand in black, antisense strand in red) to predominantly 21 and 22 nt short interfering RNAs (siRNAs). Short overhanging 3′ nucleotides, if present on the dsRNA, may be beneficial for processing of short dsRNAs. The dsRNA-processing proteins, which remain to be characterized, are represented as green and blue ovals, and assembled on the dsRNA in asymmetric fashion. In our model, this is illustrated by binding of a hypothetical blue protein or protein domain with the siRNA strand in 3′ to 5′ direction while the hypothetical green protein or protein domain is always bound to the opposing siRNA strand. These proteins or a subset remain associated with the siRNA duplex and preserve its orientation as determined by the direction of the dsRNA processing reaction. Only the siRNA sequence associated with the blue protein is able to guide target RNA cleavage. The endonuclease complex is referred to as small interfering ribonucleoprotein complex or siRNP. It is presumed here, that the endonuclease that cleaves the dsRNA may also cleave the target RNA, probably by temporarily displacing the passive siRNA strand not used for target recognition. The target RNA is then cleaved in the center of the region recognized by the sequence-complementary guide siRNA.

Example

1. Experimental Procedures

1.1 In Vitro RNAi

In vitro RNAi and lysate preparations were performed as described previously (Tuschl et al., 1999; Zamore et al., 2000). It is critical to use freshly dissolved creatine kinase (Roche) for optimal ATP regeneration. The RNAi translation assays (Fig. 1) were performed with dsRNA concentrations of 5 nM and an extended pre-incubation period of 15 min at 25°C prior to the addition of in vitro transcribed, capped and polyadenylated Pp-luc and Rr-luc reporter mRNAs. The incubation was continued for 1 h and the relative amount of Pp-luc and Rr-luc protein was analyzed using the dual luciferase assay (Promega) and a Monolight 3010C luminometer (PharMingen).

1.2 RNA Synthesis

Standard procedures were used for in vitro transcription of RNA from PCR templates carrying T7 or SP6 promoter sequences, see for example (Tuschl et al., 1998). Synthetic RNA was prepared using Expedite RNA phosphoramidites (Proligo). The 3′ adapter oligonucleotide was synthesized using dimethoxytrityl-1,4-benzenedimethanol-succinyl-aminopropyl-CPG. The oligoribonucleotides were deprotected in 3 ml of 32% ammonia/ethanol (3/1) for 4 h at 55°C (Expedite RNA) or 16 h at 55°C (3′ and 5′ adapter DNA/RNA chimeric oligonucleotides) and then desilylated and gel-purified as described previously (Tuschl et al., 1993). RNA transcripts for dsRNA preparation including long 3′ overhangs were generated from PCR templates that contained a T7 promoter in sense and an SP6 promoter in antisense direction. The transcription template for sense and antisense target RNA was PCR-amplified with GCGTAATACGACTCACTATAGAACAATTGCTTTTACAG (underlined, T7 promoter) as 5′ primer and

ATTTAGGTGACACTATAGGCATAAAGAATTGAAGA (underlined, SP6 promoter) as 3' primer and the linearized Pp-luc plasmid (pGEM-luc sequence) (Tuschl et al., 1999) as template; the T7-transcribed sense RNA was 177 nt long with the Pp-luc sequence between pos. 113-273 relative to the start codon and followed by 17 nt of the complement of the SP6 promoter sequence at the 3' end. Transcripts for blunt-ended dsRNA formation were prepared by transcription from two different PCR products which only contained a single promoter sequence.

DsRNA annealing was carried out using a phenol/chloroform extraction. Equimolar concentration of sense and antisense RNA (50 nM to 10 μM, depending on the length and amount available) in 0.3 M NaOAc (pH 6) were incubated for 30 s at 90°C and then extracted at room temperature with an equal volume of phenol/chloroform, and followed by a chloroform extraction to remove residual phenol. The resulting dsRNA was precipitated by addition of 2.5 -3 volumes of ethanol. The pellet was dissolved in lysis buffer (100 mM KCl, 30 mM HEPES-KOH, pH 7.4, 2 mM $Mg(OAc)_2$) and the quality of the dsRNA was verified by standard agarose gel electrophoreses in 1x TAE-buffer. The 52 bp dsRNAs with the 17 nt and 20 nt 3' overhangs (Figure 6) were annealed by incubating for 1 min at 95 °C, then rapidly cooled to 70°C and followed by slow cooling to room temperature over a 3 h period (50 μl annealing reaction, 1 μM strand concentration, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5). The dsRNAs were then phenol/chloroform extracted, ethanol-precipitated and dissolved in lysis buffer.

Transcription of internally ^{32}P-radiolabeled RNA used for dsRNA preparation (Figures 2 and 4) was performed using 1 mM ATP, CTP, GTP, 0.1 or 0.2 mM UTP, and 0.2-0.3 μM -^{32}P-UTP (3000 Ci/mmol), or the respective ratio for radiolabeled nucleoside triphosphates other than UTP. Labeling of the cap of the target RNAs was performed as described previously. The target RNAs were gel-purified after cap-labeling.

1.3 Cleavage Site Mapping

Standard RNAi reactions were performed by pre-incubating 10 nM dsRNA for 15 min followed by addition of 10 nM cap-labeled target RNA. The reaction was stopped after a further 2 h (Figure 2A) or 2.5 h incubation (Figure 5B and 6B) by proteinase K treatment (Tuschl et al., 1999). The samples were then analyzed on 8 or 10% sequencing gels. The 21 and 22 nt synthetic RNA duplexes were used at 100 nM final concentration (Fig 5B).

1.4 Cloning of ~21 nt RNAs

The 21 nt RNAs were produced by incubation of radiolabeled dsRNA in Drosophila lysate in absence of target RNA (200 µl reaction, 1 h incubation, 50 nM dsP111, or 100 nM dsP52 or dsP39). The reaction mixture was subsequently treated with proteinase K (Tuschl et al., 1999) and the dsRNA-processing products were separated on a denaturing 15% polyacrylamide gel. A band, including a size range of at least 18 to 24 nt, was excised, eluted into 0.3 M NaCl overnight at 4°C and in siliconized tubes. The RNA was recovered by ethanol-precipitation and dephosphorylated (30 µl reaction, 30 min, 50°C, 10 U alkaline phosphatase, Roche). The reaction was stopped by phenol/chloroform extraction and the RNA was ethanol-precipitated. The 3' adapter oligonucleotide (pUUUaaccgcatccttctcx: uppercase, RNA; lowercase, DNA; p, phosphate; x, 4-hydroxymethylbenzyl) was then ligated to the dephosphorylated ~21 nt RNA (20 µl reaction, 30 min, 37°C, 5 µM 3' adapter, 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.2 mM ATP, 0.1 mg/ml acetylated BSA, 15% DMSO, 25 U T4 RNA ligase, Amersham-Pharmacia) (Pan and Uhlenbeck, 1992). The ligation reaction was stopped by the addition of an equal volume of 8 M urea/50 mM EDTA stopmix and directly loaded on a 15% gel. Ligation yields were greater 50%. The ligation product was recovered from the gel and 5'-phosphorylated (20 µl reaction, 30 min, 37°C, 2 mM ATP, 5 U T4 polynucleotide kinase, NEB). The phosphorylation reaction was stopped by phenol/chloroform extraction and RNA was recovered by ethanol-precipitation. Next, the

5′ adapter (tactaatacgactcactAAA: uppercase, RNA; lowercase, DNA) was ligated to the phosphorylated ligation product as described above. The new ligation product was gel-purified and eluted from the gel slice in the presence of reverse transcription primer (GACTAGCTG**GAATTC**AAGGATGCGGTTAAA: bold, Eco RI site) used as carrier. Reverse transcription (15 µl reaction, 30 min, 42°C, 150 U Superscript II reverse transcriptase, Life Technologies) was followed by PCR using as 5′ primer CAGCCAACG**GAATTC**ATACGACTCACTAAA (bold, Eco RI site) and the 3′ RT primer. The PCR product was purified by phenol/chloroform extraction and ethanol-precipitated. The PCR product was then digested with Eco RI (NEB) and concatamerized using T4 DNA ligase (high conc., NEB). Concatamers of a size range of 200 to 800 bp were separated on a low-melt agarose gel, recovered from the gel by a standard melting and phenol extraction procedure, and ethanol-precipitated. The unpaired ends were filled in by incubation with Taq polymerase under standard conditions for 15 min at 72°C and the DNA product was directly ligated into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). Colonies were screened using PCR and M13-20 and M13 Reverse sequencing primers. PCR products were directly submitted for custom sequencing (Sequence Laboratories Göttingen GmbH, Germany). On average, four to five 21mer sequences were obtained per clone.

1.5 2D-TLC Analysis

Nuclease P1 digestion of radiolabeled, gel-purified siRNAs and 2D-TLC was carried out as described (Zamore et al., 2000). Nuclease T2 digestion was performed in 10 µl reactions for 3 h at 50°C in 10 mM ammonium acetate (pH 4.5) using 2 µg/µl carrier tRNA and 30 U ribonuclease T2 (Life Technologies). The migration of non-radioactive standards was determined by UV shadowing. The identity of nucleoside-3′,5′-disphosphates was confirmed by co-migration of the T2 digestion products with standards prepared by 5′-^{32}P-phosphorylation of commercial nucleoside 3′-monophosphates using γ-32P-ATP and T4 polynucleotide kinase (data not shown).

2. Results and Discussion

2.1 Length Requirements for Processing of dsRNA to 21 and 22 nt RNA Fragments

Lysate prepared from D. melanogaster syncytial embryos recapitulates RNAi in vitro providing a novel tool for biochemical analysis of the mechanism of RNAi (Tuschl et al., 1999; Zamore et al., 2000). In vitro and in vivo analysis of the length requirements of dsRNA for RNAi has revealed that short dsRNA (<150 bp) are less effective than longer dsRNAs in degrading target mRNA (Caplen et al., 2000; Hammond et al., 2000; Ngo et al., 1998); Tuschl et al., 1999). The reasons for reduction in mRNA degrading efficiency are not understood. We therefore examined the precise length requirement of dsRNA for target RNA degradation under optimized conditions in the Drosophila lysate (Zamore et al., 2000). Several series of dsRNAs were synthesized and directed against firefly luciferase (Pp-luc) reporter RNA. The specific suppression of target RNA expression was monitored by the dual luciferase assay (Tuschl et al., 1999) (Figures 1A and 1B). We detected specific inhibition of target RNA expression for dsRNAs as short as 38 bp, but dsRNAs of 29 to 36 bp were not effective in this process. The effect was independent of the target position and the degree of inhibition of Pp-luc mRNA expression correlated with the length of the dsRNA, i.e. long dsRNAs were more effective than short dsRNAs.

It has been suggested that the 21-23 nt RNA fragments generated by processing of dsRNAs are the mediators of RNA interference and co-suppression (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). We therefore analyzed the rate of 21-23 nt fragment formation for a subset of dsRNAs ranging in size between 501 to 29 bp. Formation of 21-23 nt fragments in Drosophila lysate (Figure 2) was readily detectable for 39 to 501 bp long dsRNAs but was significantly delayed for the 29 bp dsRNA. This observation is consistent with a role of 21-23 nt fragments in guiding mRNA cleavage and provides an explanation for the

lack of RNAi by 30 bp dsRNAs. The length dependence of 21-23 mer formation is likely to reflect a biologically relevant control mechanism to prevent the undesired activation of RNAi by short intramolecular base-paired structures of regular cellular RNAs.

2.2 39 bp dsRNA Mediates Target RNA Cleavage at a Single Site

Addition of dsRNA and 5′-capped target RNA to the Drosophila lysate results in sequence-specific degradation of the target RNA (Tuschl et al., 1999). The target mRNA is only cleaved within the region of identity with the dsRNA and many of the target cleavage sites were separated by 21-23 nt (Zamore et al., 2000). Thus, the number of cleavage sites for a given dsRNA was expected to roughly correspond to the length of the dsRNA divided by 21. We mapped the target cleavage sites on a sense and an antisense target RNA which was 5′ radiolabeled at the cap (Zamore et al., 2000) (Figures 3A and 3B). Stable 5′ cleavage products were separated on a sequencing gel and the position of cleavage was determined by comparison with a partial RNase T1 and an alkaline hydrolysis ladder from the target RNA.

Consistent with the previous observation (Zamore et al., 2000), all target RNA cleavage sites were located within the region of identity to the dsRNA. The sense or the antisense traget was only cleaved once by 39 bp dsRNA. Each cleavage site was located 10 nt from the 5′ end of the region covered by the dsRNA (Figure 3B). The 52 bp dsRNA, which shares the same 5′ end with the 39 bp dsRNA, produces the same cleavage site on the sense target, located 10 nt from the 5′ end of the region of identity with the dsRNA, in addition to two weaker cleavage sites 23 and 24 nt downstream of the first site. The antisense target was only cleaved once, again 10 nt from the 5′ end of the region covered by its respective dsRNA. Mapping of the cleavage sites for the 38 to 49 bp dsRNAs shown in Figure 1 showed that the first and predominant cleavage site was always located 7 to 10 nt downstream of the region covered by the dsRNA (data not

shown). This suggests that the point of target RNA cleavage is determined by the end of the dsRNA and could imply that processing to 21-23 mers starts from the ends of the duplex.

Cleavage sites on sense and antisense target for the longer 111 bp dsRNA were much more frequent than anticipated and most of them appear in clusters separated by 20 to 23 nt (Figures 3A and 3B). As for the shorter dsRNAs, the first cleavage site on the sense target is 10 nt from the 5′ end of the region spanned by the dsRNA, and the first cleavage site on the antisense target is located 9 nt from the 5′ end of region covered by the dsRNA. It is unclear what causes this disordered cleavage, but one possibility could be that longer dsRNAs may not only get processed from the ends but also internally, or there are some specificity determinants for dsRNA processing which we do not yet understand. Some irregularities to the 21-23 nt spacing were also previously noted (Zamore et al., 2000). To better understand the molecular basis of dsRNA processing and target RNA recognition, we decided to analyze the sequences of the 21-23 nt fragments generated by processing of 39, 52, and 111 bp dsRNAs in the Drosophila lysate.

2.3 dsRNA is Processed to 21 and 22 nt RNAs by an RNase III-Like Mechanism

In order to characterize the 21-23 nt RNA fragments we examined the 5′ and 3′ termini of the RNA fragments. Periodate oxidation of gel-purified 21-23 nt RNAs followed by ß-elimination indicated the presence of a terminal 2′ and 3′ hydroxyl groups. The 21-23 mers were also responsive to alkaline phosphatase treatment indicating the presence of a 5′ terminal phosphate group. The presence of 5′ phosphate and 3′ hydroxyl termini suggests that the dsRNA could be processed by an enzymatic activity similar to E. coli RNase III (for reviews, see (Dunn, 1982; Nicholson, 1999; Robertson, 1990; Robertson, 1982)).

Directional cloning of 21-23 nt RNA fragments was performed by ligation of a 3′ and 5′ adapter oligonucleotide to the purified 21-23 mers using T4 RNA ligase. The ligation products were reverse transcribed, PCR-amplified, concatamerized, cloned, and sequenced. Over 220 short RNAs were sequenced from dsRNA processing reactions of the 39, 52 and 111 bp dsRNAs (Figure 4A). We found the following length distribution: 1% 18 nt, 5% 19 nt, 12% 20 nt, 45% 21 nt, 28% 22 nt, 6% 23 nt, and 2% 24 nt. Sequence analysis of the 5′ terminal nucleotide of the processed fragments indicated that oligonucleotides with a 5′ guanosine were underrepresented. This bias was most likely introduced by T4 RNA ligase which discriminates against 5′ phosphorylated guanosine as donor oligonucleotide; no significant sequence bias was seen at the 3′ end. Many of the ~21 nt fragments derived from the 3′ ends of the sense or antisense strand of the duplexes include 3′ nucleotides that are derived from untemplated addition of nucleotides during RNA synthesis using T7 RNA polymerase. Interestingly, a significant number of endogenous Drosophila ~21 nt RNAs were also cloned, some of them from LTR and non-LTR retrotransposons (data not shown). This is consistent with a possible role for RNAi in transposon silencing.

The ~21 nt RNAs appear in clustered groups (Figure 4A) which cover the entire dsRNA sequences. Apparently, the processing reaction cuts the dsRNA by leaving staggered 3′ ends, another characteristic of RNase III cleavage. For the 39 bp dsRNA, two clusters of ~21 nt RNAs were found from each dsRNA-constituting strand including overhanging 3′ ends, yet only one cleavage site was detected on the sense and antisense target (Figures 3A and 3B). If the ~21 nt fragments were present as single-stranded guide RNAs in a complex that mediates mRNA degradation, it could be assumed that at least two target cleavage sites exist, but this was not the case. This suggests that the ~21 nt RNAs may be present in double-stranded form in the endonuclease complex but that only one of the strands can be used for target RNA recognition and cleavage. The use of

only one of the ~21 nt strands for target cleavage may simply be determined by the orientation in which the ~21 nt duplex is bound to the nuclease complex. This orientation is defined by the direction in which the original dsRNA was processed.

The ~21mer clusters for the 52 bp and 111 bp dsRNA are less well defined when compared to the 39 bp dsRNA. The clusters are spread over regions of 25 to 30 nt most likely representing several distinct subpopulations of ~21 nt duplexes and therefore guiding target cleavage at several nearby sites. These cleavage regions are still predominantly separated by 20 to 23 nt intervals. The rules determining how regular dsRNA can be processed to ~21 nt fragments are not yet understood, but it was previously observed that the approx. 21-23 nt spacing of cleavage sites could be altered by a run of uridines (Zamore et al., 2000). The specificity of dsRNA cleavage by E. coli RNase III appears to be mainly controlled by antideterminants, i.e. excluding some specific base-pairs at given positions relative to the cleavage site (Zhang and Nicholson, 1997).

To test whether sugar-, base- or cap-modification were present in processed ~21 nt RNA fragments, we incubated radiolabeled 505 bp Pp-luc dsRNA in lysate for 1 h, isolated the ~21 nt products, and digested it with P1 or T2 nuclease to mononucleotides. The nucleotide mixture was then analyzed by 2D thin-layer chromatography (Figure 4B). None of the four natural ribonucleotides were modified as indicated by P1 or T2 digestion. We have previously analyzed adenosine to inosine conversion in the ~21 nt fragments (after a 2 h incubation) and detected a small extent (<0.7%) deamination (Zamore et al., 2000); shorter incubation in lysate (1 h) reduced this inosine fraction to barely detectable levels. RNase T2, which cleaves 3' of the phosphodiester linkage, produced nucleoside 3'-phosphate and nucleoside 3',5'-diphosphate, thereby indicating the presence of a 5'-terminal monophosphate. All four nucleoside 3',5'-diphosphates were detected and suggest that the internucleotidic linkage was

cleaved with little or no sequence-specificity. In summary, the ~21 nt fragments are unmodified and were generated from dsRNA such that 5'-monophosphates and 3'-hydroxyls were present at the 5'-end.

2.4 Synthetic 21 and 22 nt RNAs Mediate Target RNA Cleavage

Analysis of the products of dsRNA processing indicated that the ~21 nt fragments are generated by a reaction with all the characteristics of an RNase III cleavage reaction (Dunn, 1982; Nicholson, 1999; Robertson, 1990; Robertson, 1982). RNase III makes two staggered cuts in both strands of the dsRNA, leaving a 3' overhang of about 2 nt. We chemically synthesized 21 and 22 nt RNAs, identical in sequence to some of the cloned ~21 nt fragments, and tested them for their ability to mediate target RNA degradation (Figures 5A and 5B). The 21 and 22 nt RNA duplexes were incubated at 100 nM concentrations in the lysate, a 10-fold higher concentrations than the 52 bp control dsRNA. Under these conditions, target RNA cleavage is readily detectable. Reducing the concentration of 21 and 22 nt duplexes from 100 to 10 nM does still cause target RNA cleavage. Increasing the duplex concentration from 100 nM to 1000 nM however does not further increase target cleavage, probably due to a limiting protein factor within the lysate.

In contrast to 29 or 30 bp dsRNAs that did not mediate RNAi, the 21 and 22 nt dsRNAs with overhanging 3' ends of 2 to 4 nt mediated efficient degradation of target RNA (duplexes 1, 3, 4, 6, Figures 5A and 5B). Blunt-ended 21 or 22 nt dsRNAs (duplexes 2, 5, and 7, Figures 5A and 5B) were reduced in their ability to degrade the target and indicate that overhanging 3' ends are critical for reconstitution of the RNA-protein nuclease complex. The single-stranded overhangs may be required for high affinity binding of the ~ 21 nt duplex to the protein components. A 5' terminal phosphate, although present after dsRNA processing, was not required to mediate target RNA cleavage and was absent from the short synthetic RNAs.

The synthetic 21 and 22 nt duplexes guided cleavage of sense as well as antisense targets within the region covered by the short duplex. This is an important result considering that a 39 bp dsRNA, which forms two pairs of clusters of ~21 nt fragments (Fig. 2), cleaved sense or antisense target only once and not twice. We interpret this result by suggesting that only one of two strands present in the ~21 nt duplex is able to guide target RNA cleavage and that the orientation of the ~21 nt duplex in the nuclease complex is determined by the initial direction of dsRNA processing. The presentation of an already perfectly processed ~21 nt duplex to the in vitro system however does allow formation of the active sequence-specific nuclease complex with two possible orientations of the symmetric RNA duplex. This results in cleavage of sense as well as antisense target within the region of identity with the 21 nt RNA duplex.

The target cleavage site is located 11 or 12 nt downstream of the first nucleotide that is complementary to the 21 or 22 nt guide sequence, i.e. the cleavage site is near center of the region covered by the 21 or 22 nt RNAs (Figures 4A and 4B). Displacing the sense strand of a 22 nt duplex by two nucleotides (compare duplexes 1 and 3 in Figure 5A) displaced the cleavage site of only the antisense target by two nucleotides. Displacing both sense and antisense strand by two nucleotides shifted both cleavage sites by two nucleotides (compare duplexes 1 and 4). We predict that it will be possible to design a pair of 21 or 22 nt RNAs to cleave a target RNA at almost any given position.

The specificity of target RNA cleavage guided by 21 and 22 nt RNAs appears exquisite as no aberrant cleavage sites are detected (Figure 5B). It should however be noted, that the nucleotides present in the 3' overhang of the 21 and 22 nt RNA duplex may contribute less to substrate recognition than the nucleotides near the cleavage site. This is based on the observation that the 3' most nucleotide in the 3' overhang of the active duplexes 1 or 3 (Figure 5A) is not complementary to the target. A detailed

analysis of the specificity of RNAi can now be readily undertaken using synthetic 21 and 22 nt RNAs.

Based on the evidence that synthetic 21 and 22 nt RNAs with overhanging 3′ ends mediate RNA interference, we propose to name the ~21 nt RNAs "short interfering RNAs" or siRNAs and the respective RNA-protein complex a "small interfering ribonucleoprotein particle" or siRNP.

2.5 3′ Overhangs of 20 nt on short dsRNAs inhibit RNAi

We have shown that short blunt-ended dsRNAs appear to be processed from the ends of the dsRNA. During our study of the length dependence of dsRNA in RNAi, we have also analyzed dsRNAs with 17 to 20 nt overhanging 3′ ends and found to our surprise that they were less potent than blunt-ended dsRNAs. The inhibitory effect of long 3′ ends was particularly pronounced for dsRNAs up to 100 bp but was less dramatic for longer dsRNAs. The effect was not due to imperfect dsRNA formation based on native gel analysis (data not shown). We tested if the inhibitory effect of long overhanging 3′ ends could be used as a tool to direct dsRNA processing to only one of the two ends of a short RNA duplex.

We synthesized four combinations of the 52 bp model dsRNA, blunt-ended, 3′ extension on only the sense strand, 3′-extension on only the antisense strand, and double 3′ extension on both strands, and mapped the target RNA cleavage sites after incubation in lysate (Figures 6A and 6B). The first and predominant cleavage site of the sense target was lost when the 3′ end of the antisense strand of the duplex was extended, and vice versa, the strong cleavage site of the antisense target was lost when the 3′ end of sense strand of the duplex was extended. 3′ Extensions on both strands rendered the 52 bp dsRNA virtually inactive. One explanation for the dsRNA inactivation by ~20 nt 3′ extensions could be the association of single-stranded RNA-binding proteins which could interfere with the association of one of the dsRNA-processing factors at this end. This result

is also consistent with our model where only one of the strands of the siRNA duplex in the assembled siRNP is able to guide target RNA cleavage. The orientation of the strand that guides RNA cleavage is defined by the direction of the dsRNA processing reaction. It is likely that the presence of 3′ staggered ends may facilitate the assembly of the processing complex. A block at the 3′ end of the sense strand will only permit dsRNA processing from the opposing 3′ end of the antisense strand. This in turn generates siRNP complexes in which only the antisense strand of the siRNA duplex is able to guide sense target RNA cleavage. The same is true for the reciprocal situation.

The less pronounced inhibitory effect of long 3′ extensions in the case of longer dsRNAs (≥500 bp, data not shown) suggests to us that long dsRNAs may also contain internal dsRNA-processing signals or may get processed cooperatively due to the association of multiple cleavage factors.

2.6 A Model for dsRNA-Directed mRNA Cleavage

The new biochemical data update the model for how dsRNA targets mRNA for destruction (Figure 7). Double-stranded RNA is first processed to short RNA duplexes of predominantly 21 and 22 nt in length and with staggered 3′ ends similar to an RNase III-like reaction (Dunn, 1982; Nicholson, 1999; Robertson, 1982). Based on the 21-23 nt length of the processed RNA fragments it has already been speculated that an RNase III-like activity may be involved in RNAi (Bass, 2000). This hypothesis is further supported by the presence of 5′ phosphates and 3′ hydroxyls at the termini of the siRNAs as observed in RNase III reaction products (Dunn, 1982; Nicholson, 1999). Bacterial RNase III and the eukaryotic homologs Rnt1p in S. cerevisiae and Pac1p in S. pombe have been shown to function in processing of ribosomal RNA as well as snRNA and snoRNAs (see for example Chanfreau et al., 2000).

Little is known about the biochemistry of RNase III homologs from plants, animals or human. Two families of RNase III enzymes have been identified predominantly by database-guided sequence analysis or cloning of cDNAs. The first RNase III family is represented by the 1327 amino acid long D. melanogaster protein drosha (Acc. AF116572). The C-terminus is composed of two RNase III and one dsRNA-binding domain and the N-terminus is of unknown function. Close homologs are also found in C. elegans (Acc. AF160248) and human (Acc. AF189011) (Filippov et al., 2000; Wu et al., 2000). The drosha-like human RNase III was recently cloned and characterized (Wu et al., 2000). The gene is ubiquitously expressed in human tissues and cell lines, and the protein is localized in the nucleus and the nucleolus of the cell. Based on results inferred from antisense inhibition studies, a role of this protein for rRNA processing was suggested. The second class is represented by the C. elegans gene K12H4.8 (Acc. S44849) coding for a 1822 amino acid long protein. This protein has an N-terminal RNA helicase motif which is followed by 2 RNase III catalytic domains and a dsRNA-binding motif, similar to the drosha RNase III family. There are close homologs in S. pombe (Acc. Q09884), A. thaliana (Acc. AF187317), D. melanogaster (Acc. AE003740), and human (Acc. AB028449) (Filippov et al., 2000; Jacobsen et al., 1999; Matsuda et al., 2000). Possibly the K12H4.8 RNase III/helicase is the likely candidate to be involved in RNAi.

Genetic screens in C. elegans identified rde-1 and rde-4 as essential for activation of RNAi without an effect on transposon mobilization or co-suppression (Dernburg et al., 2000; Grishok et al., 2000; Ketting and Plasterk, 2000; Tabara et al., 1999). This led to the hypothesis that these genes are important for dsRNA processing but are not involved in mRNA target degradation. The function of both genes is as yet unknown, the rde-1 gene product is a member of a family of proteins similar to the rabbit protein eIF2C (Tabara et al., 1999), and the sequence of rde-4 has not yet

been described. Future biochemical characterization of these proteins should reveal their molecular function.

Processing to the siRNA duplexes appears to start from the ends of both blunt-ended dsRNAs or dsRNAs with short (1-5 nt) 3′ overhangs, and proceeds in approximately 21-23 nt steps. Long (~20 nt) 3′ staggered ends on short dsRNAs suppress RNAi, possibly through interaction with single-stranded RNA-binding proteins. The suppression of RNAi by single-stranded regions flanking short dsRNA and the lack of siRNA formation from short 30 bp dsRNAs may explain why structured regions frequently encountered in mRNAs do not lead to activation of RNAi.

Without wishing to be bound by theory, we presume that the dsRNA-processing proteins or a subset of these remain associated with the siRNA duplex after the processing reaction. The orientation of the siRNA duplex relative to these proteins determines which of the two complementary strands functions in guiding target RNA degradation. Chemically synthesized siRNA duplexes guide cleavage of sense as well as antisense target RNA as they are able to associate with the protein components in either of the two possible orientation.

The remarkable finding that synthetic 21 and 22 nt siRNA duplexes can be used for efficient mRNA degradation provides new tools for sequence-specific regulation of gene expression in functional genomics as well as biomedical studies. The siRNAs may be effective in mammalian systems where long dsRNAs cannot be used due to the activation of the PKR response (Clemens, 1997). As such, the siRNA duplexes represent a new alternative to antisense or ribozyme therapeutics.

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Claims

1. Isolated double-stranded RNA molecule, wherein each RNA strand has a length from 19-23 nucleotides, wherein said RNA molecule is capable of target-specific nucleic acid modifications.

2. The RNA molecule of claim 1 wherein at least one strand has a 3'-overhang from 1-5 nucleotides.

3. The RNA molecule of claim 1 or 2 capable of target-specific RNA interference and/or DNA methylation.

4. The RNA molecule of any one of claims 1-3, wherein each strand has a length from 20-22 nucleotides.

5. The RNA molecule of any one of claims 2-4, wherein the 3'-overhang is from 1-3 nucleotides.

6. The RNA molecule of any one of claims 2-5, wherein the 3'-overhang is stabilized against degradation.

7. The RNA molecule of any one of claims 1-6, which contains at least one modified nucleotide analogue.

8. The RNA molecule of claim 7, wherein the modified nucleotide analogue is selected from sugar- or backbone modified ribonucleotides.

9. The RNA molecule according to claim 7 or 8, wherein the nucleotide analogue is a sugar-modified ribonucleotide, wherein the 2'-OH group is replaced by a group selected from H, OR, R, halo, SH, SR^1,

NH_2, NHR, NR_2 or CN, wherein R is C_1-C_6 alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I.

10. The RNA molecule of claim 7 or 8, wherein the nucleotide analogue is a backbone-modified ribonucleotide containing a phosphothioate group.

11. The RNA molecule of any one of claims 1-10, which has a sequence having an identity of at least 50 percent to a predetermined mRNA target molecule.

12. The RNA molecule of claim 11, wherein the identity is at least 70 percent.

13. A method of preparing a double-stranded RNA molecule of any one of claims 1-12 comprising the steps:

(a) synthesizing two RNA strands each having a length from 19-23 nucleotides, wherein said RNA strands are capable of forming a double-stranded RNA molecule,

(b) combining the synthesized RNA strands under conditions, wherein a double-stranded RNA molecule is formed, which is capable of target-specific nucleic acid modifications.

14. The method of claim 13, wherein the RNA strands are chemically synthesized.

15. The method of claim 13, wherein the RNA strands are enzymatically synthesized.

16. A method of mediating target-specific nucleic acid modifications in a cell or an organism comprising the steps:

(a) contacting said cell or organism with the double-stranded RNA molecule of any one of claims 1-12 and

(b) mediating a target-specific nucleic acid modification effected by the double-stranded RNA towards a target nucleic acid having a sequence portion substantially corresponding to the double-stranded RNA.

17. The method of claim 16, wherein the nucleic acid modification is RNA interference and/or DNA methylation.

18. Use of the method of claim 16 or 17 for determining the function of a gene in a cell or an organism.

19. Use of the method of claim 16 or 17 for modulating the function of a gene in a cell or an organism.

20. The use of claim 16 or 17, wherein the gene is associated with a pathological condition.

21. The use of claim 20, wherein the gene is a pathogen-associated gene.

22. The use of claim 21, wherein the gene is a viral gene.

23. The use of claim 20, wherein the gene is a tumor-associated gene.

24. The use of claim 20, wherein the gene is an autoimmune disease-associated gene.

25. Pharmaceutical composition containing as an active agent at least one double-stranded RNA molecule of any one of claims 1-12 and a pharmaceutical carrier.

26. The composition of claim 25 for diagnostic applications.

27. The composition of claim 25 for therapeutic applications.

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Abstract

Double-stranded RNA (dsRNA) induces sequence-specific post-transcriptional gene silencing in many organisms by a process known as RNA interference (RNAi). Using a Drosophila in vitro system, we demonstrate that 19-23 nt short RNA fragments are the sequence-specific mediators of RNAi. The short interfering RNAs (siRNAs) are generated by an RNase III-like processing reaction from long dsRNA. Chemically synthesized siRNA duplexes with overhanging 3' ends mediate efficient target RNA cleavage in the lysate, and the cleavage site is located near the center of the region spanned by the guiding siRNA. Furthermore, we provide evidence that the direction of dsRNA processing determines whether sense or antisense target RNA can be cleaved by the produced siRNP complex.

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FIGURE 1A

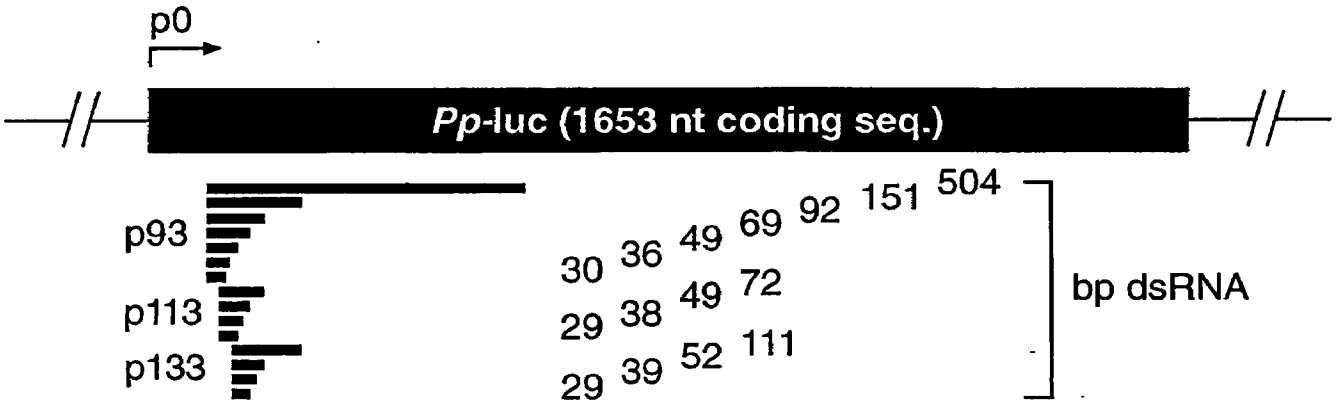


FIGURE 1B

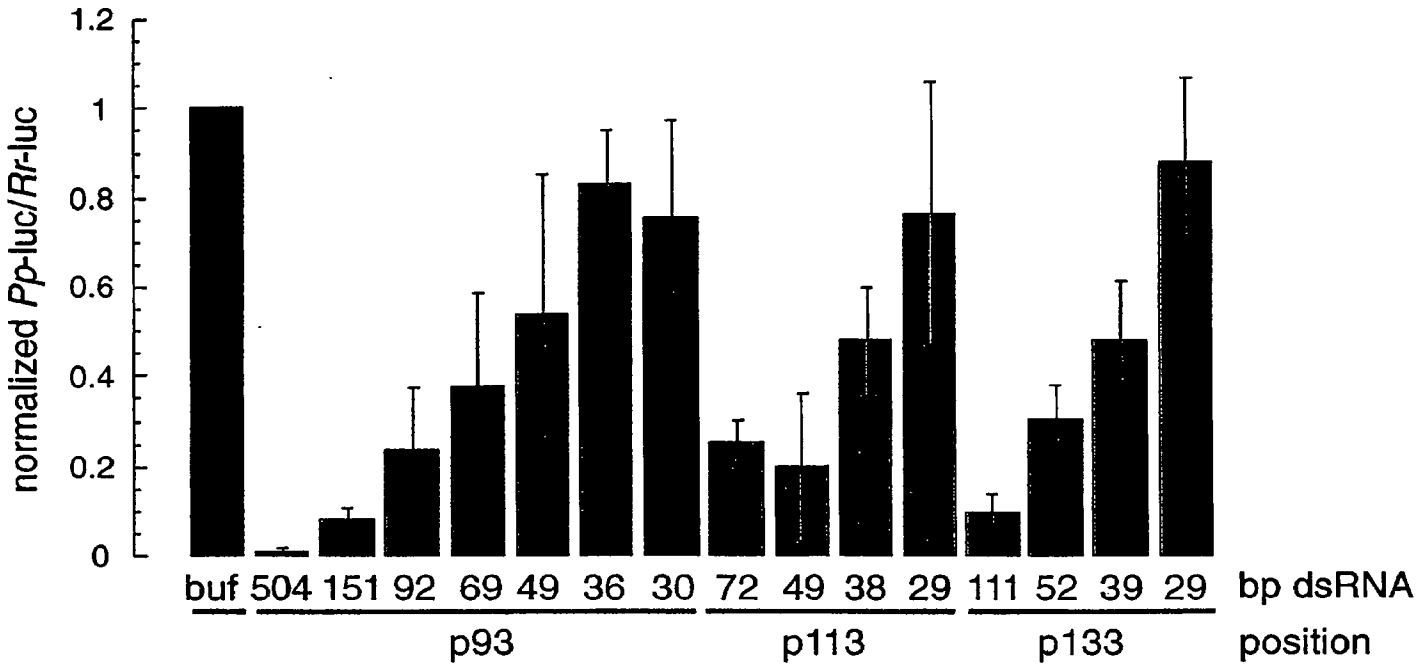


FIGURE 2

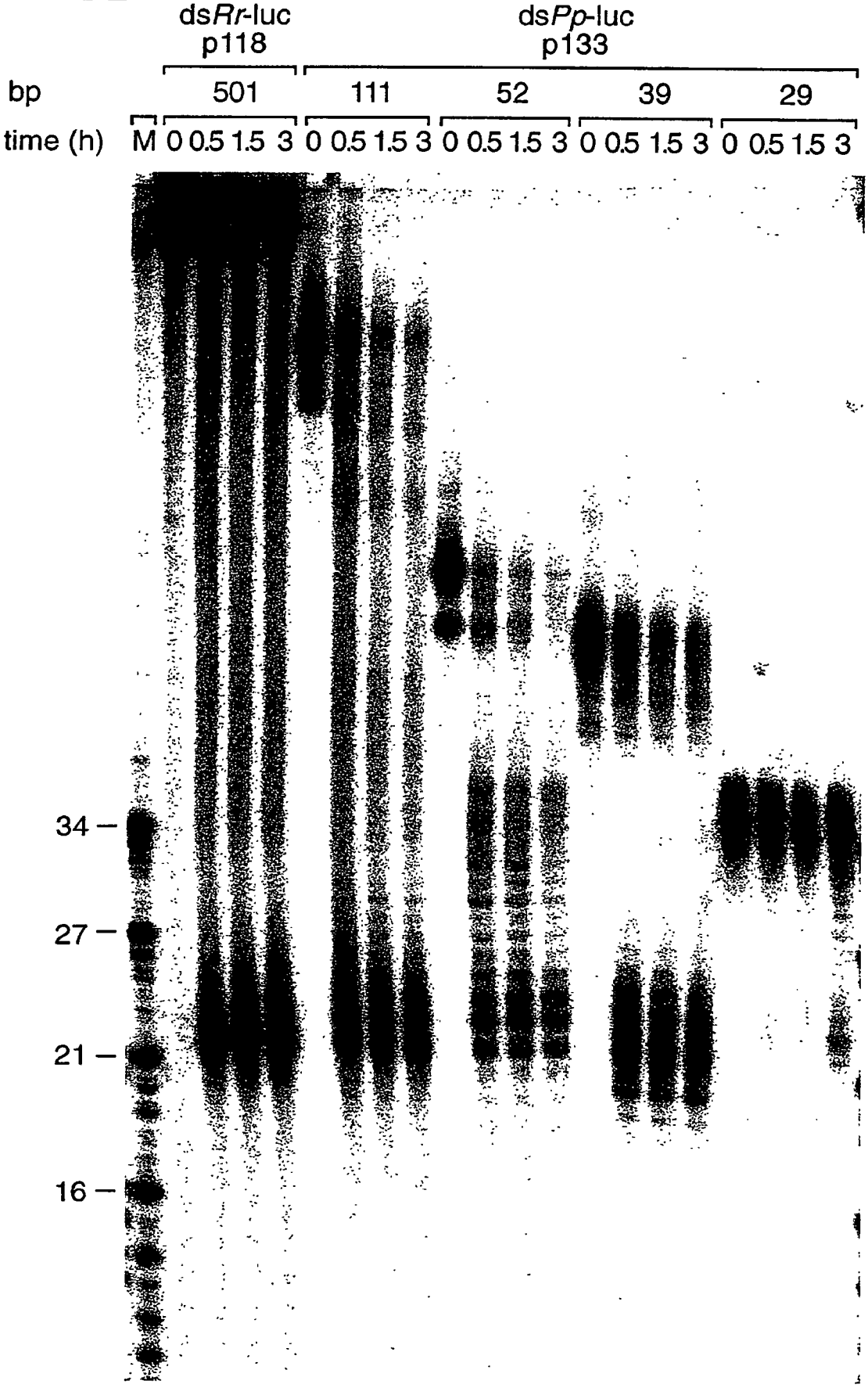

dsRr-luc
p118
dsPp-luc
p133
bp
501
111
52
39
29
time (h)
M 0 0.5 1.5 3 0 0.5 1.5 3 0 0.5 1.5 3 0 0.5 1.5 3 0 0.5 1.5 3
34
27
21
16

FIGURE 3A

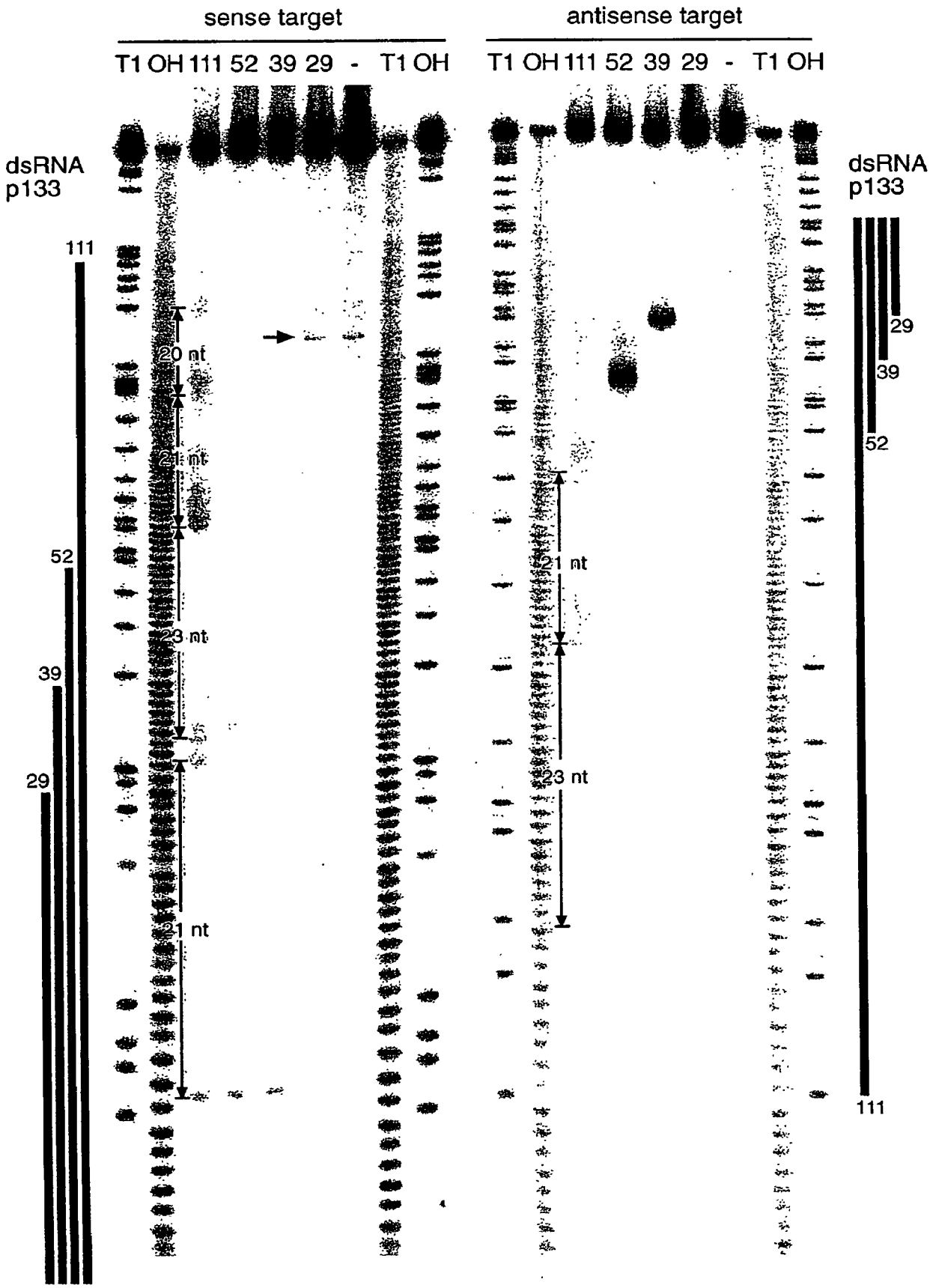

sense target
antisense target
T1 OH 111 52 39 29 - T1 OH
T1 OH 111 52 39 29 - T1 OH
dsRNA p133
dsRNA p133
111
52
39
29
20 nt
21 nt
23 nt
21 nt
21 nt
23 nt
29
39
52
111

FIGURE 3B

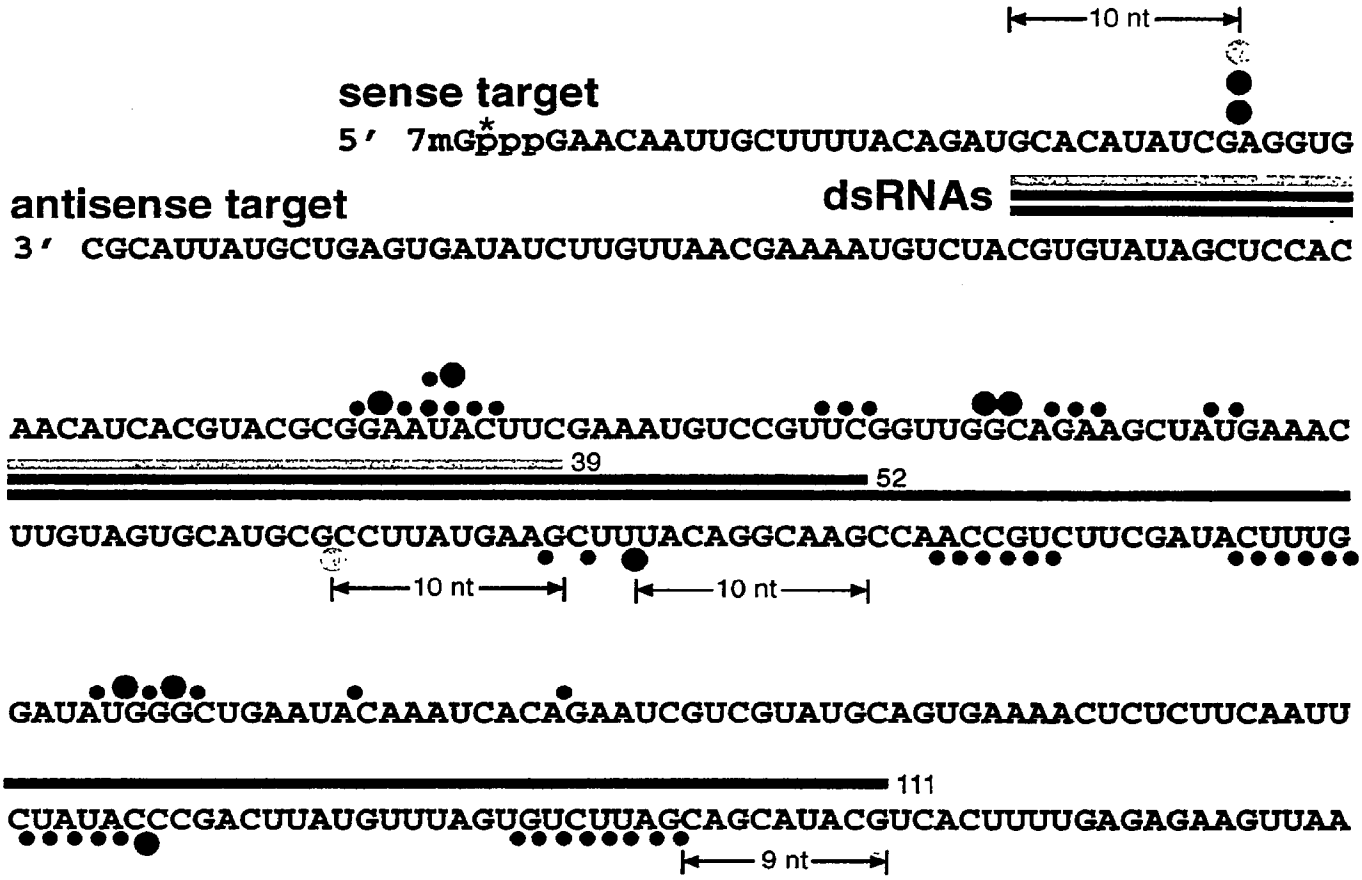
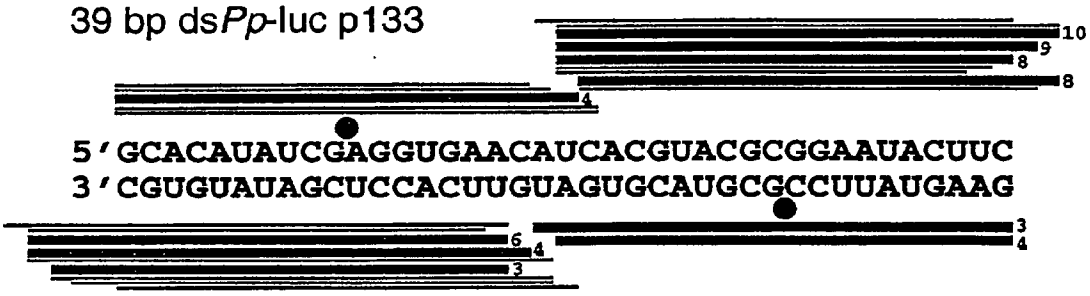
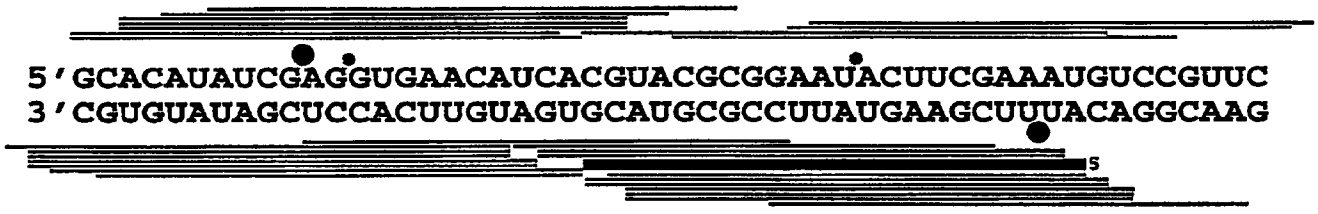

10 nt
sense target
5′ 7mG*pppGAACAAUUGCUUUUACAGAUGCACAUAUCGAGGUG
antisense target
dsRNAs
3′ CGCAUUAUGCUGAGUGAUAUCUUGUUAACGAAAAUGUCUACGUGUAUAGCUCCAC
AACAUCACGUACGCGGAAUACUUCGAAAUGUCCGUUCGGUUGGCAGAAGCUAUGAAAC
39
52
UUGUAGUGCAUGCGCCUUAUGAAGCUUUACAGGCAAGCCAACCGUCUUCGAUACUUUG
10 nt
10 nt
GAUAUGGGCUGAAUACAAAUCACAGAAUCGUCGUAUGCAGUGAAAACUCUCUUCAAUU
111
CUAUACCCGACUUAUGUUUAGUGUCUUAGCAGCAUACGUCACUUUUGAGAGAAGUUAA
9 nt
CUUUAUGCCUAUAGUGUCACCUAAAU 3′
GAAAUACGGppp*Gm7 5′

FIGURE 4A

39 bp ds*Pp*-luc p133



52 bp ds*Pp*-luc p133



111 bp ds*Pp*-luc p133

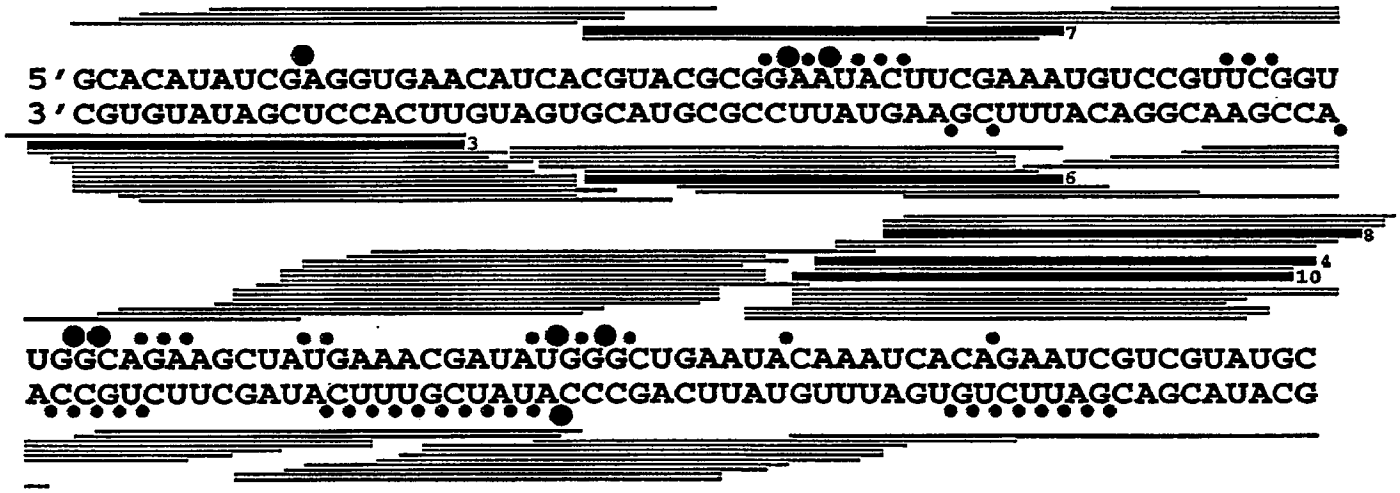


FIGURE 4B

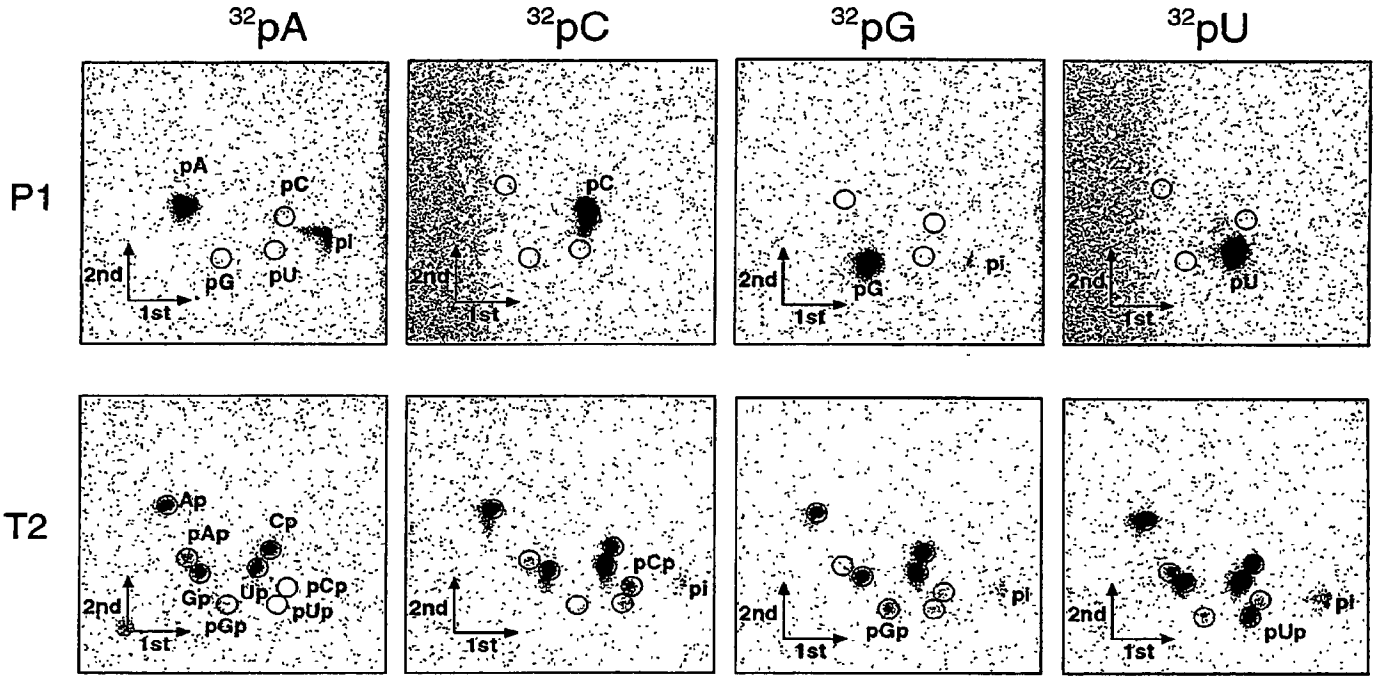

32pA
32pC
32pG
32pU
P1
T2
pA
pC
pi
pG
pU
2nd
1st
Ap
pAp
Cp
Gp
Up
pCp
pUp
pGp

FIGURE 5A

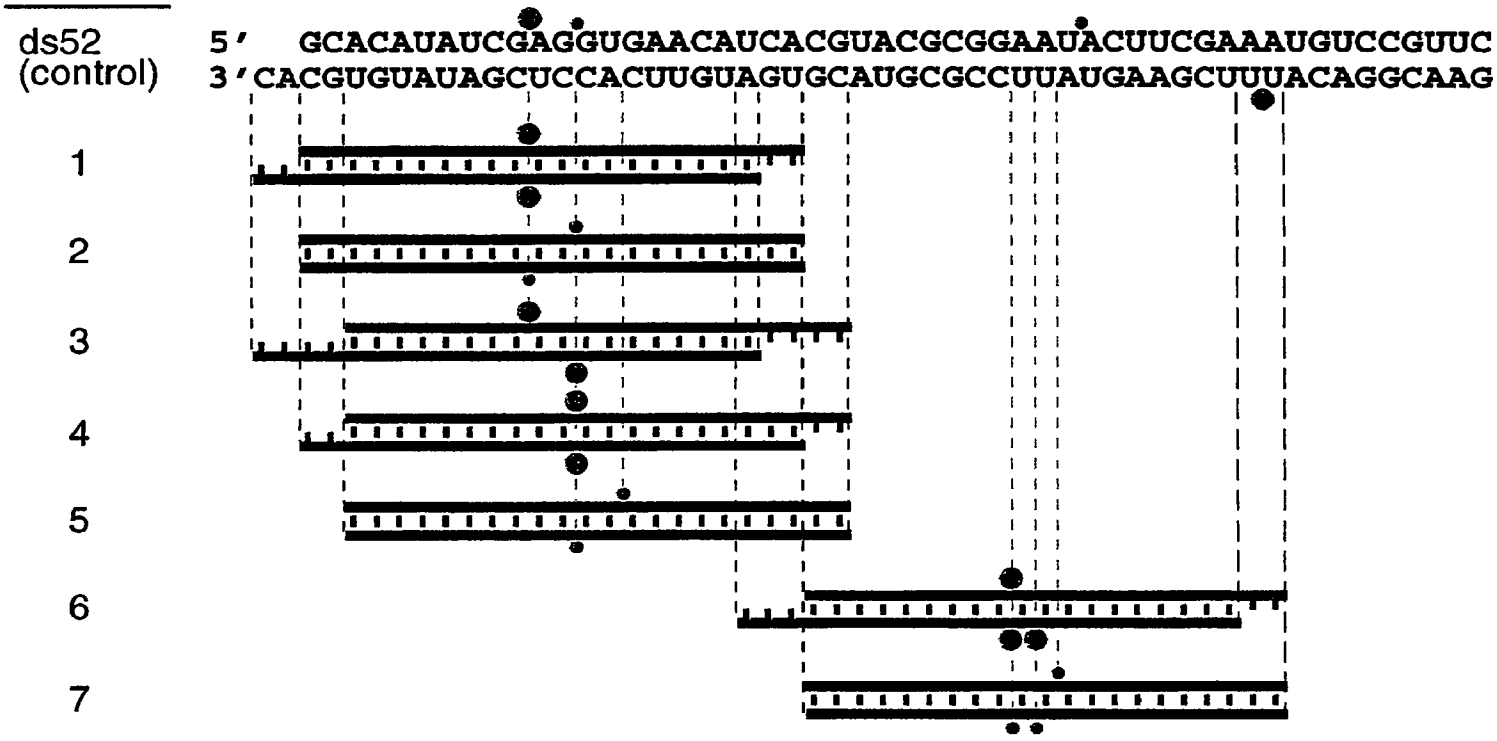

siRNAs/
dsRNA
ds52
(control)
5′ GCACAUAUCGAGGUGAACAUCACGUACGCGGAAUACUUCGAAAUGUCCGUUC
3′ CACGUGUAUAGCUCCACUUGUAGUGCAUGCGCCUUAUGAAGCUUUACAGGCAAG
1
2
3
4
5
6
7

FIGURE 5B

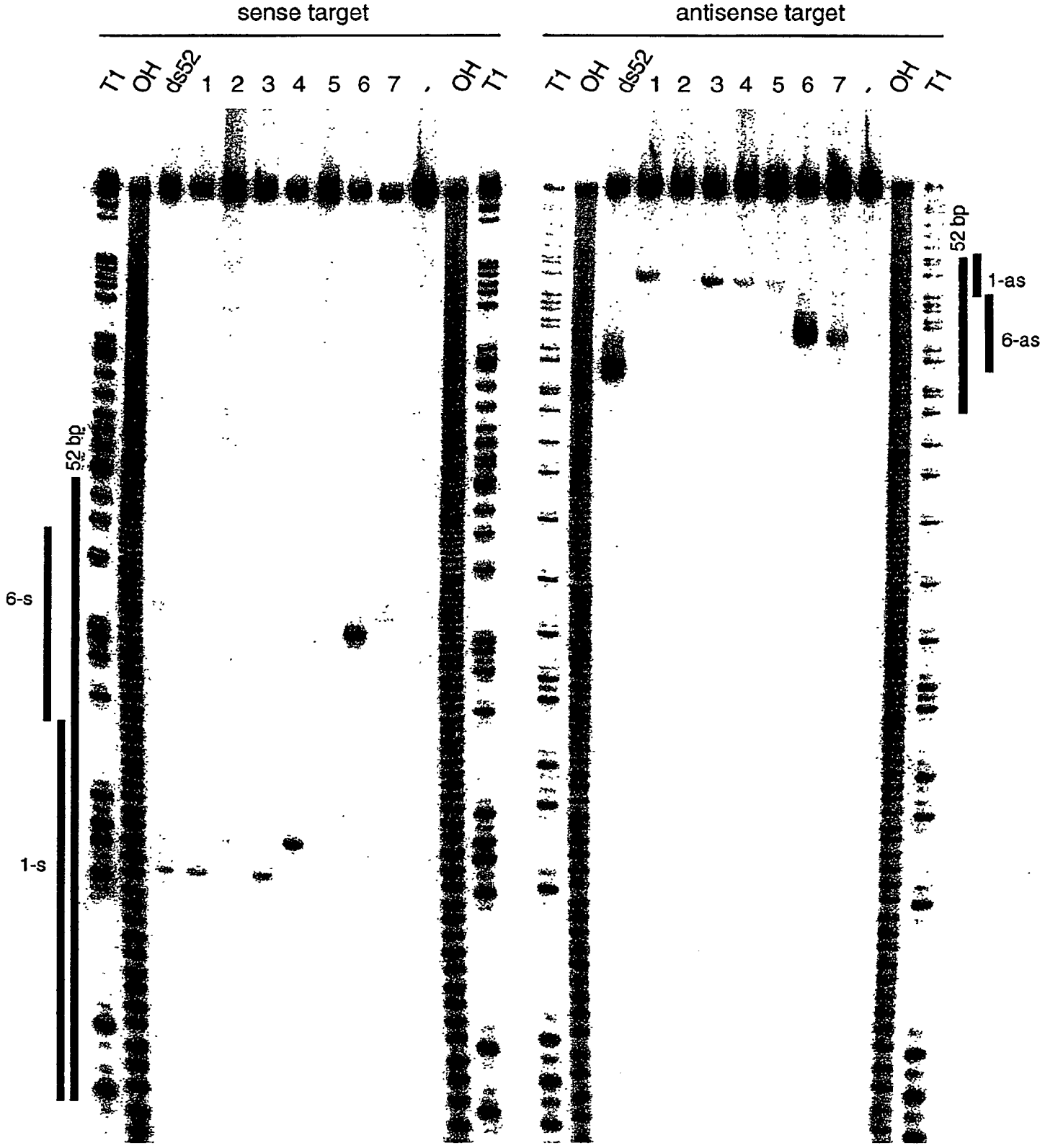

sense target
antisense target
T1
OH
ds52
1
2
3
4
5
6
7
OH
T1
52 bp
6-s
1-s
1-as
6-as

FIGURE 6A

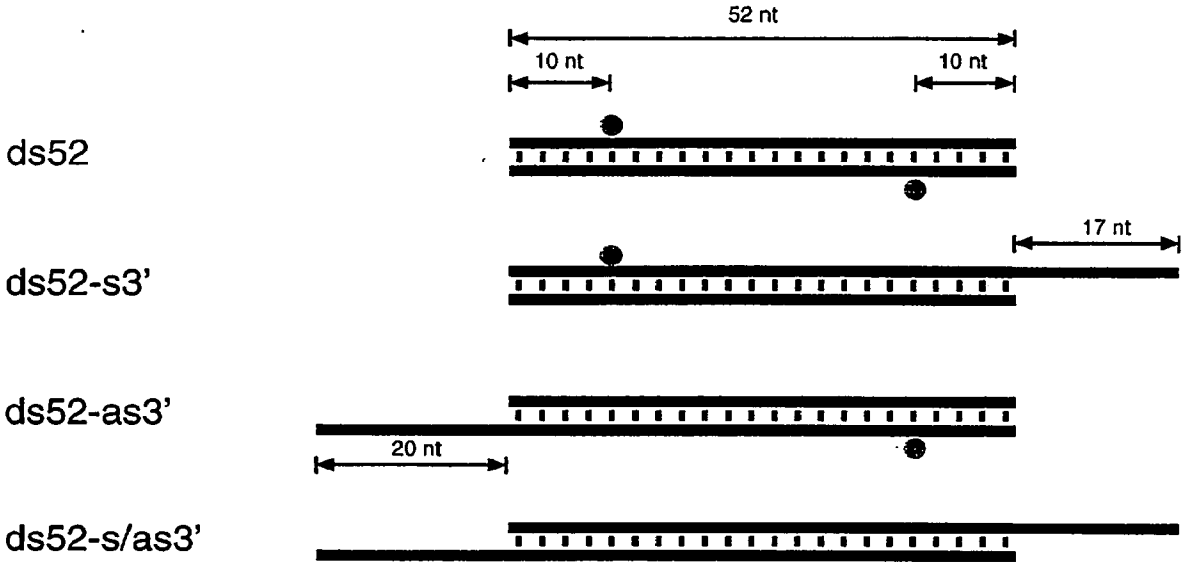


FIGURE 6B

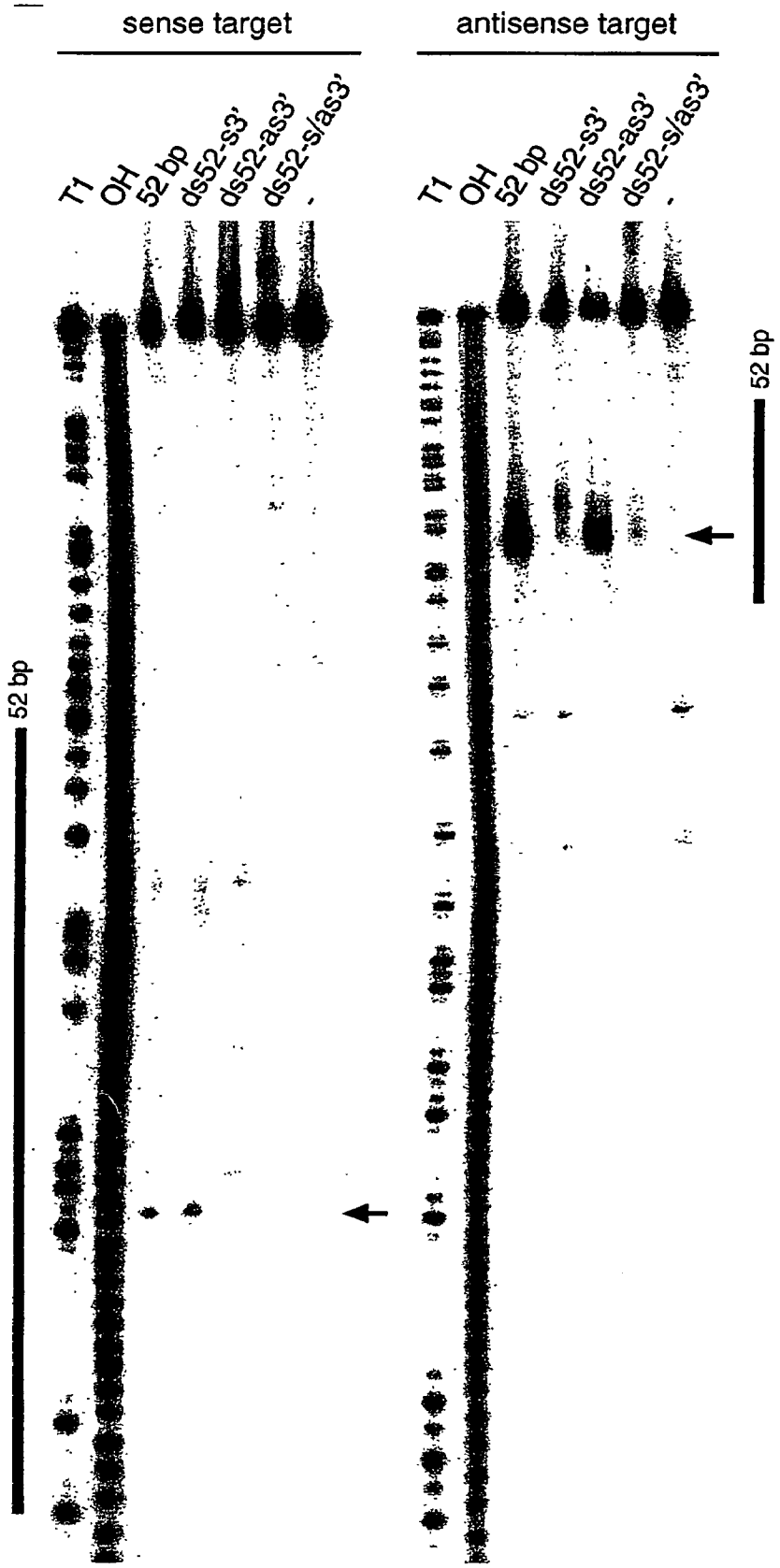

sense target
antisense target
T1
OH
52 bp
ds52-s3'
ds52-as3'
ds52-s/as3'
52 bp
52 bp

FIGURE 7

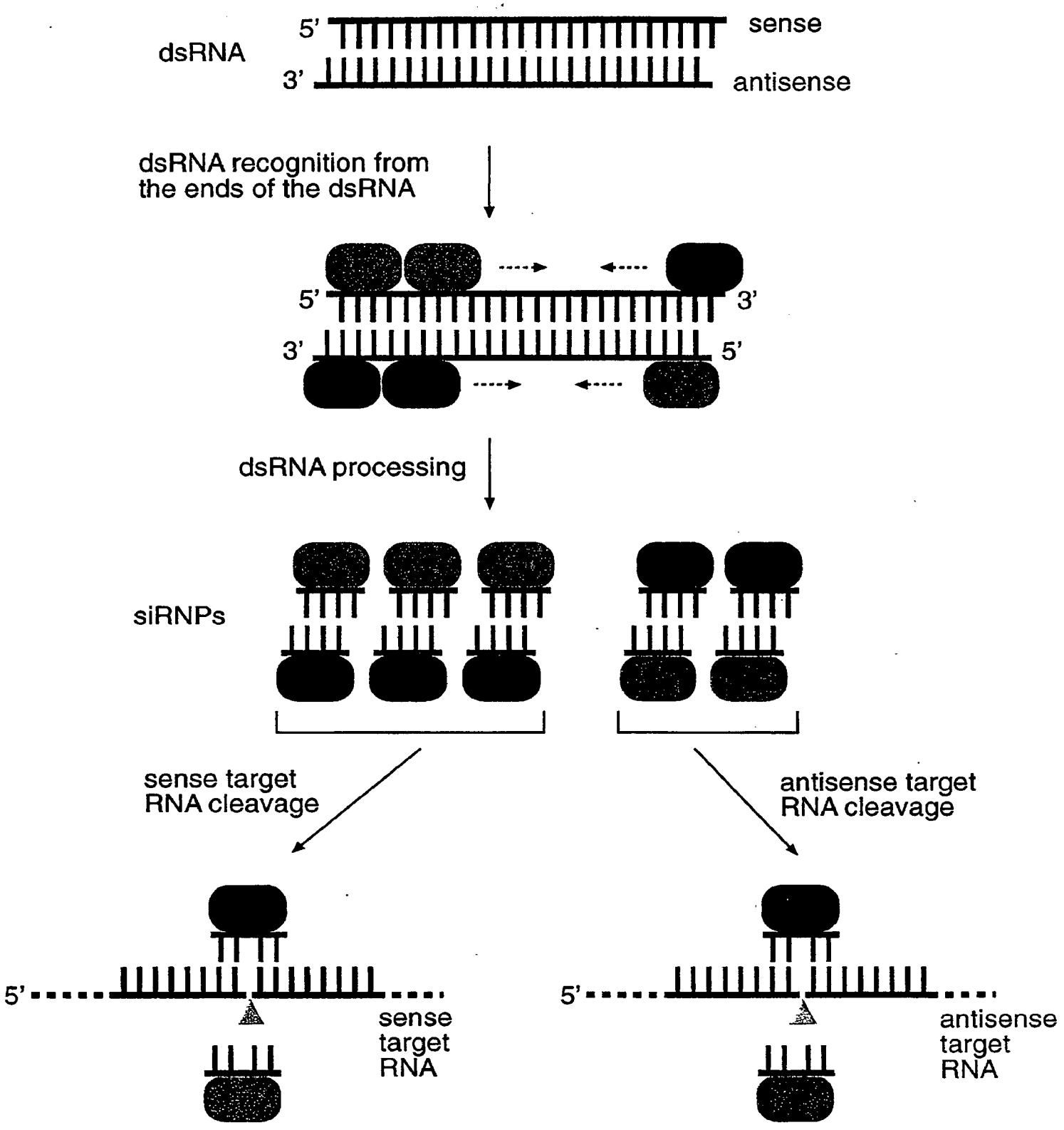

dsRNA
5'
3'
sense
antisense
dsRNA recognition from the ends of the dsRNA
5'
3'
3'
5'
dsRNA processing
siRNPs
sense target RNA cleavage
antisense target RNA cleavage
5'
sense target RNA
5'
antisense target RNA

EXHIBIT 8

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Firma:	Whitehead Institut
Bereich/Abteilung:	
zu Händen:	**Ms Patricia Granahan**
Komplette Anzahl der Seiten:	3

GI 2716 ZTM

Dear Patricia,

I'd like to inform you today about a statement from our patent attorney W. Weiss regarding to the relationship of our commonly owned patent application "Tuschl I" and the MPG/EMBL patent application "Tuschl II". We heard some rumors from one of the licensees that their might be a broader overlap of both applications, due to the co-filing of both applications on March 30, 2001. We think the statement from W. Weiss give a very clear picture of the relationship and the resulting claims. Please feel free to contact me at any time if you have further questions.

Best regards

Garching Innovation GmbH

i.A. Dr. Torsten Mummenbrauer

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DIPL.-FORSTW. DR. J. LACHNIT

IHR ZEICHEN / YOUR REF.
0707-2782 ZTM
0716

UNSER ZEICHEN / OUR REF.
23801P EP-WO/WWld

DATUM / DATE
13.11.2003

European Patent Application No. 01 985 833.1
(based on PCT/EP01/13968 of November 29, 2001)
RNA Interference is mediated by 21 and 22nt Small RNAs

1/077 3.R
2/3 a1

Dear Dr. Mummenbrauer,

Please find enclosed a short statement describing the history concerning the relationship between the patent applications Tuschl I (WO 01/75164) of MPG/Whitehead/MIT/U-Mass and Tuschl II (WO 02/44321) of MPG/EMBL.

On December 01, 2000, we filed a European patent application No. EP 00126325.0 claiming the use of double-stranded RNA molecules having overhangs for RNA interference.

On March 08, 2003, Mrs. Granahan from Whitehead Institute contacted us by telephone in order to discuss the further proceedings with regard to the Tuschl/siRNA applications.

On March 09, 2003 and March 12, 2003, the subject was discussed with Mrs. Granahan in detail on telephone. Mrs. Granahan did suggest that both applications should be combined, i.e. the content of the Tuschl II European priority application should be included in the text of the Tuschl I application.

After consultation with yourselves, we made the following suggestions to Mrs. Granahan:

1. The Tuschl I application based on a priority of March 30, 2000 and the Tuschl II application based on a priority of December 01, 2000 shall be prosecuted separately. The reason for these proceedings are substantially based on the fact that the first Tuschl I priority does not contain any indication with regard to the 3' overhangs. Further, the length ranges of the RNA molecules are narrower in Tuschl I.

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2. In order to eliminate possible prior art rejections during subsequent examination procedures, it was concluded that priorities should be claimed crosswise, if possible.

3. New data from Dr. Tuschl (Suppression of reporter gene expression by siRNAs in mammalian tissue cultures) can be included into the Tuschl I PCT-application, which had to be filed until March 30, 2001.

4. Simultaneously, the new Tuschl data shall be included into the Tuschl II PCT-application, which had to be filed until December 01, 2001 at the latest.

5. In order to separate basis for priority an additional US-Provisional application US 60/279,661 was filed on March 30, 2001.

6. Due to the agreements with Whitehead, the content of the Tuschl I and II applications is as follows:

WO 01/75164 (PCT Tuschl I) claims priorities from March 30, 2000 and January 31, 2001 and additionally from December 01, 2000 (Tuschl II priority). Further, the Tuschl I PCT-application contains the first Tuschl mammalian data.

Tuschl II PCT-application (WO 02/44321) claims priorities from December 01, 2000 (EP 00126325.0), and March 30, 2001 (US 60/279661 and PCT/US01/10118-Tuschl I PCT).

Based on the above, it is evident that claims relating to double-stranded siRNA molecules having overhangs are exclusively the subject-matter of the Tuschl II application.

Very truly yours,

Dr. W. Weiss

EXHIBIT 9

PCT/EP 01 / 13968

11 Feb. 2002

REC'D 15 MAR 2002
WIPO PCT

PA 493758

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

November 19, 2001

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***60/279,661***
FILING DATE: ***March 30, 2001***

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

P. R. GRANT
Certifying Officer

Atty. Dkt. No. 017853/0141

jc803 U.S. PTO 03/30/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

jc017 U.S. PTO 60/279661 03/30/01

Applicant: Thomas TUSCHL

Title: RNA INTERFERENCE MEDIATING SMALL RNA MOLECULES

Appl. No.: Unassigned

Filing Date: March 30, 2001

Examiner: Unknown

Art Unit: Unknown

60279661 = 033001

PROVISIONAL PATENT APPLICATION TRANSMITTAL

Commissioner for Patents
Box PROVISIONAL PATENT APPLICATION
Washington, D.C. 20231

Sir:

Transmitted herewith for filing under 37 C.F.R. § 1.53(c) is the provisional patent application of:

Thomas TUSCHL
Keplerstrasse 9
D-37085 Goettingen
Federal Republic of Germany

[X] Applicant claims small entity status under 37 CFR 1.27.

Enclosed are:

[X] Specification, Claim(s), and Abstract (45 pages).

[X] Informal drawings (14 sheets, Figures 1A-10).

[] Application Data Sheet (37 CFR 1.76).

Atty. Dkt. No. 017853/0141

The filing fee is calculated below:

		Rate		Fee Totals
Basic Fee		$150.00		$150.00
[X]	Small Entity Fees Apply (subtract ½ of above):		=	$75.00
	TOTAL FILING FEE:		=	$75.00

[X] A check in the amount of $75.00 to cover the filing fee is enclosed.

[] The required filing fees are not enclosed but will be submitted in response to the Notice to File Missing Parts of Application.

[X] The Assistant Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Assistant Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741.

Please direct all correspondence to the undersigned attorney or agent at the address indicated below.

Respectfully submitted,

Date: March 30, 2001

By _____

Richard L. Schwaab
Attorney for Applicant
Registration No. 25,479

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EXHIBIT 5

RCV BY:FOLEY & LARDNER DC : 3-29- 1 : 9:53AM : +49 89 45563 999→FOLEY & LARDNER DC C:# 6

RNA Interference Mediating Small RNA molecules

Description

The present invention relates to sequence and structural features of double-stranded (ds)RNA molecules required to mediate target-specific nucleic acid modifications such as RNA-interference and/or DNA methylation.

The term "RNA interference" (RNAi) was coined after the discovery that injection of dsRNA into the nematode C. elegans leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA (Fire et al., 1998). RNAi was subsequently also observed in insects, frogs (Oelgeschlager et al., 2000), and other animals including mice (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000) and is likely to also exist in human. RNAi is closely linked to the post-transcriptional gene-silencing (PTGS) mechanism of co-suppression in plants and quelling in fungi (Catalanotto et al., 2000; Cogoni and Macino, 1999; Dalmay et al., 2000; Ketting and Plasterk, 2000; Mourrain et al., 2000; Smardon et al., 2000) and some components of the RNAi machinery are also necessary for post-transcriptional silencing by co-suppression (Catalanotto et al., 2000; Dernburg et al., 2000; Ketting and Plasterk, 2000). The topic has also been reviewed recently (Bass, 2000; Bosher and Labouesse, 2000; Fire, 1999; Plasterk and Ketting, 2000; Sharp, 1999; Sijen and Kooter, 2000), see also the entire issue of Plant Molecular Biology, vol. 43, issue 2/3, (2000).

In plants, in addition to PTGS, introduced transgenes can also lead to transcriptional gene silencing via RNA-directed DNA methylation of cytosines (see references in Wassenegger, 2000). Genomic targets as short as 30 bp are methylated in plants in an RNA-directed manner (Pelissier, 2000). DNA methylation is also present in mammals.

The natural function of RNAi and co-suppression appears to be protection of the genome against invasion by mobile genetic elements such as retro-transposons and viruses which produce aberrant RNA or dsRNA in the host cell when they become active (Jensen et al, 1999; Ketting et al., 1999; Ratcliff et al., 1999; Tabara et al., 1999). Specific mRNA degradation prevents transposon and virus replication although some viruses are able to overcome or prevent this process by expressing proteins that suppress PTGS (Lucy et al., 2000; Voinnet et al., 2000).

DsRNA triggers the specific degradation of homologous RNAs only within the region of identity with the dsRNA (Zamore et al., 2000). The dsRNA is processed to 21-23 nt RNA fragments and the target RNA cleavage sites are regularly spaced 21-23 nt apart. It has therefore been suggested that the 21-23 nt fragments are the guide RNAs for target recognition (Zamore et al., 2000). These short RNAs were also detected in extracts prepared from D. melanogaster Schneider 2 cells which were transfected with dsRNA prior to cell lysis (Hammond et al., 2000), however, the fractions that displayed sequence-specific nuclease activity also contained a large fraction of residual dsRNA. The role of the 21-23 nt fragments in guiding mRNA cleavage is further supported by the observation that 21-23 nt fragments isolated from processed dsRNA are able, to some extent, to mediate specific mRNA degradation (Zamore et al., 2000). RNA molecules of similar size also accumulate in plant tissue that exhibits PTGS (Hamilton and Baulcombe, 1999).

Here, we use the established Drosophila in vitro system (Tuschl et al., 1999; Zamore et al., 2000) to further explore the mechanism of RNAi. We demonstrate that short 21 and 22 nt RNAs, when base-paired with 3' overhanging ends, act as the guide RNAs for sequence-specific mRNA degradation. Short 30 bp dsRNAs are unable to mediate RNAi because they are no longer processed to 21 and 22 nt RNAs. Furthermore, we defined the target RNA cleavage sites relative to the 21 and 22 nt short

interfering RNAs (siRNAs) and provide evidence that the direction of dsRNA processing determines whether a sense or an antisense target RNA can be cleaved by the produced siRNP endonuclease complex. Further, the siRNAs may also be important tools for transcriptional modulating, e.g. silencing of mammalian genes by guiding DNA methylation.

The object underlying the present invention is to provide novel agents capable of mediating target-specific RNA interference or other target-specific nucleic acid modifications such as DNA methylation, said agents having an improved efficacy and safety compared to prior art agents.

The solution of this problem is provided by an isolated double-stranded RNA molecule, wherein each RNA strand has a length from 19-23 nucleotides, wherein said RNA molecule is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation. Preferably at least one strand has a 3'-overhang from 1-5 nucleotides. The other strand may be blunt-ended or has up to 6 nucleotides 3' overhang. Also, if both strands of the dsRNA are exactly 21 or 22 nt, it is possible to observe some RNA interference when both ends are blunt (0 nt overhang). The RNA molecule is preferably a synthetic RNA molecule which is substantially free from contaminants occurring in cell extracts, e.g. from Drosophila embryos. Further, the RNA molecule is preferably substantially free from any non-target-specific contaminants, particularly non-target-specific RNA molecules e.g. from contaminants occuring in cell extracts.

Surprisingly, it was found that synthetic short double-stranded RNA molecules particularly with overhanging 3'-ends are sequence-specific mediators of RNAi and mediate efficient target-RNA cleavage, wherein the cleavage site is located near the center of the region spanned by the guiding short RNA.

Preferably, each strand of the RNA molecule has a length from 20-22 nucleotides, wherein the length of each strand may be the same or different. Preferably, the length of the 3'-overhang reaches from 1-3 nucleotides, wherein the length of the overhang may be the same or different for each strand. The RNA-strands preferably have 3'-hydroxyl groups. The 5'-terminus preferably comprises a phosphate, diphosphate, triphosphate or hydroxyl group. The most effective dsRNAs are composed of two 21 nt strands which are paired such that 1-3, particularly 2 nt 3' overhangs are present on both ends of the dsRNA. Pyrimidine nucleotides in the 3' overhang are more effective in mediating RNA interference than purine nucleotides. Also, the nucleotides present in the 3' overhang do not seem to contribute to the specificity of target RNA recognition.

Surprisingly, the double-stranded RNA molecules of the present invention exhibit a high in vivo stability in serum or in growth medium for cell cultures. In order to further enhance the stability, the 3'-overhangs may be stablized against degradation, e.g. they may be selected such that they consist of purine nucleotides, particularly adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g. Substitution of uridine 2 nt 3' overhangs by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNA interference. The absence of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium.

In an especially preferred embodiment of the present invention the RNA molecule may contain at least one modified nucleotide analogue. The nucleotide analogues may be located at positions where the target-specific activity, e.g. the RNAi mediating activity is not substantially effected, e.g. in a region at the 5'-end and/or the 3'-end of the double-stranded RNA molecule. Particularly, the overhangs may be stabilized by incorporating modified nucleotide analogues.

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Preferred nucleotide analogues are selected from sugar- or backbone-modified ribonucleotides. It should be noted, however, that also nucleobase-modified ribonucleotides, i.e. ribonucleotides, containing a non-naturally occurring nucleobase instead of a naturally occurring nucleobase such as uridines or cytidines modified at the 5-position, e.g. 5-(2-amino)propyl uridine, 5-bromo uridine; adenosines and guanosines modified at the 8-position, e.g. 8-bromo guanosine; deaza nucleotides, e.g. 7-deaza-adenosine; O- and N-alkylated nucleotides, e.g. N6-methyl adenosine are suitable. In preferred sugar-modified ribonucleotides the 2' OH-group is replaced by a group selected from H, OR, R, halo, SH, SR, NH_2, NHR, NR_2 or CN, wherein R is C_1-C_6 alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I. In preferred backbone-modified ribonucleotides the phosphoester group connecting to adjacent ribonucleotides is replaced by a modified group, e.g. of phosphothioate group. It should be noted that the above modifications may be combined.

The sequence of the double-stranded RNA molecule of the present invention has to have a sufficient identity to a nucleic acid target molecule in order to mediate target-specific RNAi and/or DNA methylation. Preferably, the sequence has an identity of at least 50%, particularly of at least 70% to the desired target molecule in the double-stranded portion of the RNA molecule. More preferably, the identity is at least 70% and most preferably 100% in the double-stranded portion of the RNA molecule. The identity of a double-stranded RNA molecule to a predetermined nucleic acid target molecule, e.g. an mRNA target molecule may be determined as follows:

$$I = \frac{n}{L} \times 100$$

wherein I is the identity in percent, n is the number of identical nucleotides in the double-stranded portion of the ds RNA and the target and L is the length of the sequence overlap of the double-stranded portion of the dsRNA and the target.

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The double-stranded RNA molecule of the invention may be prepared by a method comprising the steps:

(a) synthesizing two RNA strands each having a length from 19-23 nucleotides, wherein said RNA strands are capable of forming a double-stranded RNA molecule, wherein preferably at least one strand has a 3'-overhang from 1-5 nucleotides,

(b) combining the synthesized RNA strands under conditions, wherein a double-stranded RNA molecule is formed, which is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation.

Methods of synthesizing RNA molecules are known in the art. In this context, it is particularly referred to chemical synthesis methods as described in Verma and Eckstein (1998).

The single-stranded RNAs can also be prepared by enzymatic transcription from synthetic DNA templates or from DNA plasmids isolated from recombinant bacteria. Typically, phage RNA polymerases are used such as T7, T3 or SP6 RNA polymerase (Milligan and Uhlenbeck (1989)).

A further aspect of the present invention relates to a method of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation in a cell or an organism comprising the steps:

(a) contacting the cell or organism with the double-stranded RNA molecule of the invention and

(b) mediating a target-specific nucleic acid modificiation effected by the double-stranded RNA towards a target nucleic acid having a

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sequence portion substantially corresponding to the double-stranded RNA.

The method of the invention may be used for determining the function of a gene in a cell or an organism or even for modulating the function of a gene in a cell or an organism, being capable of mediating RNA interference. The cell is preferably a eukaryotic cell or a cell line, e.g. a plant cell or an animal cell, such as a mammalian cell, e.g. an embryonic cell, a pluripotent stem cell, a tumor cell, e.g. a teratocarcinoma cell or a virus-infected cell. The organism is preferably a eukaryotic organism, e.g. a plant or an animal, such as a mammal, particularly a human.

The target gene to which the RNA molecule of the invention is directed may be associated with a pathological condition. For example, the gene may be a pathogen-associated gene, e.g. a viral gene, a tumor-associated gene or an autoimmune disease-associated gene. The target gene may also be a heterologous gene expressed in a recombinant cell or a genetically altered organism. By determinating or modulating, particularly, inhibiting the function of such a gene valuable information and therapeutic benefits in the agricultural field or in the medicine or veterinary medicine field may be obtained.

The dsRNA is usually administered as a pharmaceutical composition. The administration may be carried out by known methods, wherein a nucleic acid is introduced into a desired target cell in vitro or in vivo. Commonly used gene transfer techniques include calcium phosphate, DEAE-dextran, electroporation and microinjection and viral methods (Graham, F.L. and van der Eb, A.J. (1973) Virol. 52, 456; McCutchan, J.H. and Pagano, J.S. (1968), J. Natl. Cancer Inst. 41, 351; Chu, G. et al (1987), Nucl. Acids Res. 15, 1311; Fraley, R. et al. (1980), J. Biol. Chem. 255, 10431; Capecchi, M.R. (1980), Cell 22, 479). A recent addition to this arsenal of techniques for the introduction of DNA into cells is the use of cationic liposomes

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(Felgner, P.L. et al. (1987), Proc. Natl. Acad. Sci USA 84, 7413). Commercially available cationic lipid formulations are e.g. Tfx 50 (Promega) or Lipofectamin2000 (Life Technologies).

Thus, the invention also relates to a pharmaceutical composition containing as an active agent at least one double-stranded RNA molecule as described above and a pharmaceutical carrier. The composition may be used for diagnostic and for therapeutic applications.

For diagnostic or therapeutic applications, the composition may be in form of a solution, e.g. an injectable solution, a cream, ointment, tablet, suspension or the like. The composition may be administered in any suitable way, e.g. by injection, by oral, topical, nasal, rectal application etc. The carrier may be any suitable pharmaceutical carrier. Preferably, a carrier is used, which is capable of increasing the efficacy of the RNA molecules to enter the target-cells. Suitable examples of such carriers are liposomes, particularly cationic liposomes.

Further, the present invention is explained in more detail in the following figures and examples.

Figure Legends

Figure 1: Double-stranded RNA as short as 38 bp can mediate RNAi. (A) Graphic representation of dsRNAs used for targeting Pp-luc mRNA. Three series of blunt-ended dsRNAs covering a range of 29 to 504 bp were prepared. The position of the first nucleotide of the sense strand of the dsRNA is indicated relative to the start codon of Pp-luc mRNA (p1). (B) RNA interference assay (Tuschl et al., 1999). Ratios of target Pp-luc to control Rr-luc activity were normalized to a buffer control (black bar). DsRNAs (5 nM) were preincubated in Drosophila lysate for 15 min at 25°C prior to the addition of 7-methyl-guanosine-capped Pp-luc and Rr-luc

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mRNAs (~50 pM). The incubation was continued for another hour and then analyzed by the dual luciferase assay (Promega). The data are the average from at least four independent experiments ± standard deviation.

Figure 2: A 29 bp dsRNA is no longer processed to 21-23 nt fragments. Time course of 21-23 mer formation from processing of internally ^{32}P-labeled dsRNAs (5 nM) in the Drosophila lysate. The length and source of the dsRNA are indicated. An RNA size marker (M) has been loaded in the left lane and the fragment sizes are indicated. Double bands at time zero are due to incompletely denatured dsRNA.

Figure 3: Short dsRNAs cleave the mRNA target only once.
(A) Denaturing gel electrophoreses of the stable 5' cleavage products produced by 1 h incubation of 10 nM sense or antisense RNA ^{32}P-labeled at the cap with 10 nM dsRNAs of the p133 series in Drosophila lysate. Length markers were generated by partial nuclease T1 digestion and partial alkaline hydrolysis (OH) of the cap-labeled target RNA. The regions targeted by the dsRNAs are indicated as black bars on both sides. The 20-23 nt spacing between the predominant cleavage sites for the 111 bp long dsRNA is shown. The horizontal arrow indicates unspecific cleavage not due to RNAi. (B) Position of the cleavage sites on sense and antisense target RNAs. The sequences of the capped 177 nt sense and 180 nt antisense target RNAs are represented in antiparallel orientation such that complementary sequence are opposing each other. The region targeted by the different dsRNAs are indicated by differently colored bars positioned between sense and antisense target sequences. Cleavage sites are indicated by circles: large circle for strong cleavage, small circle for weak cleavage. The ^{32}P-radiolabeled phosphate group is marked by an asterisk.

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Figure 4: 21 and 22 *nt* RNA fragments are generated by an RNase III-like mechanism.

(A) Sequences of ~21 nt RNAs after dsRNA processing. The ~21 nt RNA fragments generated by dsRNA processing were directionally cloned and sequenced. Oligoribonucleotides originating from the sense strand of the dsRNA are indicated as blue lines, those originating from the antisense strand as red lines. Thick bars are used if the same sequence was present in multiple clones, the number at the right indicating the frequency. The target RNA cleavage sites mediated by the dsRNA are indicated as orange circles, large circle for strong cleavage, small circle for weak cleavage (see Figure 3B). Circles on top of the sense strand indicated cleavage sites within the sense target and circles at the bottom of the dsRNA indicate cleavage site in the antisense target. Up to five additional nucleotides were identified in ~21 nt fragments derived from the 3' ends of the dsRNA. These nucleotides are random combinations of predominantly C, G, or A residues and were most likely added in an untemplated fashion during T7 transcription of the dsRNA-constituting strands. (B) Two-dimensional TLC analysis of the nucleotide composition of ~21 nt RNAs. The ~21 nt RNAs were generated by incubation of internally radiolabeled 504 bp Pp-luc dsRNA in Drosophila lysate, gel-purified, and then digested to mononucleotides with nuclease P1 (top row) or ribonuclease T2 (bottom row). The dsRNA was internally radiolabeled by transcription in the presence of one of the indicated α-^{32}P nucleoside triphosphates. Radioactivity was detected by phosphorimaging. Nucleoside 5'-monophosphates, nucleoside 3'-monophosphates, nucleoside 5',3'-diphosphates, and inorganic phosphate are indicated as pN, Np, pNp, and p_i, respectively. Black circles indicate UV-absorbing spots from non-radioactive carrier nucleotides. The 3',5'-bisphosphates (red circles) were identified by co-migration with radiolabeled standards prepared by 5'-phosphorylation of nucleoside 3'-monophosphates with T4 polynucleotide kinase and γ-^{32}P-ATP.

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Figure 5: Synthetic 21 and 22 nt RNAs Mediate Target RNA Cleavage. (A) Graphic representation of control 52 bp dsRNA and synthetic 21 and 22 nt dsRNAs. The sense strand of 21 and 22 nt short interfering RNAs (siRNAs) is shown blue, the antisense strand in red. The sequences of the siRNAs were derived from the cloned fragments of 52 and 111 bp dsRNAs (Figure 4A), except for the 22 nt antisense strand of duplex 5. The siRNAs in duplex 6 and 7 were unique to the 111 bp dsRNA processing reaction. The two 3' overhanging nucleotides indicated in green are present in the sequence of the synthetic antisense strand of duplexes 1 and 3. Both strands of the control 52 bp dsRNA were prepared by in vitro transcription and a fraction of transcripts may contain untemplated 3' nucleotide addition. The target RNA cleavage sites directed by the siRNA duplexes are indicated as orange circles (see legend to Figure 4A) and were determined as shown in Figure 5B. (B) Position of the cleavage sites on sense and antisense target RNAs. The target RNA sequences are as described in Figure 3B. Control 52 bp dsRNA (10 nM) or 21 and 22 nt RNA duplexes 1-7 (100 nM) were incubated with target RNA for 2.5 h at 25°C in Drosophila lysate. The stable 5' cleavage products were resolved on the gel. The cleavage sites are indicated in Figure 5A. The region targeted by the 52 bp dsRNA or the sense (s) or antisense (as) strands are indicated by the black bars to the side of the gel. The cleavage sites are all located within the region of identity of the dsRNAs. For precise determination of the cleavage sites of the antisense strand, a lower percentage gel was used.

Figure 6: Long 3' overhangs on short dsRNAs inhibit RNAi. (A) Graphic representation of 52 bp dsRNA constructs. The 3' extensions of sense and antisense strand are indicated in blue and red, respectively. The observed cleavage sites on the target RNAs are represented as orange circles analogous to Figure 4A and were determined as shown in Figure 6B. (B) Position of the cleavage sites on sense and antisense target RNAs. The target RNA sequences are as described in Figure 3B. DsRNA (10 nM) was incubated with target RNA for 2.5 h at 25°C in Drosophila lysate. The

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stable 5' cleavage products were resolved on the gel. The major cleavage sites are indicated with a horizontal arrow and also represented in Figure 6A. The region targeted by the 52 bp dsRNA is represented as black bar at both sides of the gel.

Figure 7: Proposed Model for RNAi.

RNAi is predicted to begin with processing of dsRNA (sense strand in black, antisense strand in red) to predominantly 21 and 22 nt short interfering RNAs (siRNAs). Short overhanging 3' nucleotides, if present on the dsRNA, may be beneficial for processing of short dsRNAs. The dsRNA-processing proteins, which remain to be characterized, are represented as green and blue ovals, and assembled on the dsRNA in asymmetric fashion. In our model, this is illustrated by binding of a hypothetical blue protein or protein domain with the siRNA strand in 3' to 5' direction while the hypothetical green protein or protein domain is always bound to the opposing siRNA strand. These proteins or a subset remain associated with the siRNA duplex and preserve its orientation as determined by the direction of the dsRNA processing reaction. Only the siRNA sequence associated with the blue protein is able to guide target RNA cleavage. The endonuclease complex is referred to as small interfering ribonucleoprotein complex or siRNP. It is presumed here, that the endonuclease that cleaves the dsRNA may also cleave the target RNA, probably by temporarily displacing the passive siRNA strand not used for target recognition. The target RNA is then cleaved in the center of the region recognized by the sequence-complementary guide siRNA.

Figure 8: Reporter constructs and siRNA duplexes.

(a) The firefly (Pp-luc) and sea pansy (Rr-luc) luciferase reporter gene regions from plasmids pGL2-Control, pGL-3-Control and pRL-TK (Promega) are illustrated. SV40 regulatory elements, the HSV thymidine kinase promoter and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase

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expression from pGL2 is approx. 10-fold lower than from pGL3 in transfected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes. (b) The sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2, GL3 and RL luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only 3 single nucleotide substitutions (boxed in gray). As unspecific control, a duplex with the inverted GL2 sequence, invGL2, was synthesized. The 2 nt 3' overhang of 2'-deoxythymidine is indicated as TT; uGL2 is similar to GL2 siRNA but contains ribo-uridine 3' overhangs.

Figure 9: RNA interference by siRNA duplexes.
Ratios of target control luciferase were normalized to a buffer control (bu, black bars); gray bars indicate ratios of *Photinus pyralis* (Pp-luc) GL2 or GL3 luciferase to *Renilla reniformis* (Rr-luc) RL luciferase (left axis), white bars indicate RL to GL2 or GL3 ratios (right axis). Panels a, c, e, g and i describe experiments performed with the combination of pGL2-Control and pRL-TK reporter plasmids, panels b, d, f, h and j with pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of Pp-luc/Rr-luc for the buffer control (bu) varied between 0.5 and 10 for pGL2/pRL and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and between the various cell lines tested. The plotted data were averaged from three independent experiments ± S.D.

Figure 10: Effects of 21 nt siRNA, 50 bp and 500 bp dsRNAs on luciferase expression in HeLa cells.
The exact length of the long dsRNAs is indicated below the bars. Panels a, c and e describe experiments performed with pGL2-Control and pRL-TK reporter plasmids, panels b, d and f with pGL3-Control and pRL-TK reporter plasmids. The data were averaged from two independent experiments ± S.D. (a), (b) Absolute Pp-luc expression, plotted in arbitrary luminescence units. (c), (d) Rr-luc expression, plotted in arbitrary luminescence units. (e),

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(f) Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (bu, black bars); the luminescence ratios for 50 or 500 bp dsRNAs were normalized to the respective ratios observed for 50 and 500 bp dsRNA from humanized GFP (hG, black bars). It should be noted that the overall differences in sequences between the 49 and 484 bp dsRNAs targeting GL2 and GL3 are not sufficient to confer specificity between GL2 and GL3 targets (43 nt uninterrupted identity in 49 bp segment, 239 nt longest uninterrupted identity in 484 bp segment).

Example 1

RNA Interference Mediated by Small Synthetic RNAs

1. Experimental Procedures

1.1 In Vitro RNAi

In vitro RNAi and lysate preparations were performed as described previously (Tuschl et al., 1999; Zamore et al., 2000). It is critical to use freshly dissolved creatine kinase (Roche) for optimal ATP regeneration. The RNAi translation assays (Fig. 1) were performed with dsRNA concentrations of 5 nM and an extended pre-incubation period of 15 min at 25°C prior to the addition of in vitro transcribed, capped and polyadenylated Pp-luc and Rr-luc reporter mRNAs. The incubation was continued for 1 h and the relative amount of Pp-luc and Rr-luc protein was analyzed using the dual luciferase assay (Promega) and a Monolight 3010C luminometer (PharMingen).

1.2 RNA Synthesis

Standard procedures were used for in vitro transcription of RNA from PCR templates carrying T7 or SP6 promoter sequences, see for example (Tuschl et al., 1998). Synthetic RNA was prepared using Expedite RNA phosphoramidites (Proligo). The 3' adapter oligonucleotide was synthesized using

dimethoxytrityl-1,4-benzenedimethanol-succinyl-aminopropyl-CPG. The oligoribonucleotides were deprotected in 3 ml of 32% ammonia/ethanol (3/1) for 4 h at 55°C (Expedite RNA) or 16 h at 55°C (3' and 5' adapter DNA/RNA chimeric oligonucleotides) and then desilylated and gel-purified as described previously (Tuschl et al., 1993). RNA transcripts for dsRNA preparation including long 3' overhangs were generated from PCR templates that contained a T7 promoter in sense and an SP6 promoter in antisense direction. The transcription template for sense and antisense target RNA was PCR-amplified with GCGTAATACGACTCACTATAGAACAATTGCTTTTACAG (underlined, T7 promoter) as 5' primer and ATTTAGGTGACACTATAGGCATAAAGAATTGAAGA (underlined, SP6 promoter) as 3' primer and the linearized Pp-luc plasmid (pGEM-luc sequence) (Tuschl et al., 1999) as template; the T7-transcribed sense RNA was 177 nt long with the Pp-luc sequence between pos. 113-273 relative to the start codon and followed by 17 nt of the complement of the SP6 promoter sequence at the 3' end. Transcripts for blunt-ended dsRNA formation were prepared by transcription from two different PCR products which only contained a single promoter sequence.

DsRNA annealing was carried out using a phenol/chloroform extraction. Equimolar concentration of sense and antisense RNA (50 nM to 10 μM, depending on the length and amount available) in 0.3 M NaOAc (pH 6) were incubated for 30 s at 90°C and then extracted at room temperature with an equal volume of phenol/chloroform, and followed by a chloroform extraction to remove residual phenol. The resulting dsRNA was precipitated by addition of 2.5-3 volumes of ethanol. The pellet was dissolved in lysis buffer (100 mM KCl, 30 mM HEPES-KOH, pH 7.4, 2 mM $Mg(OAc)_2$) and the quality of the dsRNA was verified by standard agarose gel electrophoreses in 1 x TAE-buffer. The 52 bp dsRNAs with the 17 nt and 20 nt 3' overhangs (Figure 6) were annealed by incubating for 1 min at 95 °C, then rapidly cooled to 70°C and followed by slow cooling to room temperature

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over a 3 h period (50 µl annealing reaction, 1 µM strand concentration, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5). The dsRNAs were then phenol/chloroform extracted, ethanol-precipitated and dissolved in lysis buffer.

Transcription of internally ^{32}P-radiolabeled RNA used for dsRNA preparation (Figures 2 and 4) was performed using 1 mM ATP, CTP, GTP, 0.1 or 0.2 mM UTP, and 0.2-0.3 µM -^{32}P-UTP (3000 Ci/mmol), or the respective ratio for radiolabeled nucleoside triphosphates other than UTP. Labeling of the cap of the target RNAs was performed as described previously. The target RNAs were gel-purified after cap-labeling.

1.3 Cleavage Site Mapping

Standard RNAi reactions were performed by pre-incubating 10 nM dsRNA for 15 min followed by addition of 10 nM cap-labeled target RNA. The reaction was stopped after a further 2 h (Figure 2A) or 2.5 h incubation (Figure 5B and 6B) by proteinase K treatment (Tuschl et al., 1999). The samples were then analyzed on 8 or 10% sequencing gels. The 21 and 22 nt synthetic RNA duplexes were used at 100 nM final concentration (Fig 5B).

1.4 Cloning of ~21 nt RNAs

The 21 nt RNAs were produced by incubation of radiolabeled dsRNA in Drosophila lysate in absence of target RNA (200 µl reaction, 1 h incubation, 50 nM dsP111, or 100 nM dsP52 or dsP39). The reaction mixture was subsequently treated with proteinase K (Tuschl et al., 1999) and the dsRNA-processing products were separated on a denaturing 15% polyacrylamide gel. A band, including a size range of at least 18 to 24 nt, was excised, eluted into 0.3 M NaCl overnight at 4°C and in siliconized tubes. The RNA was recovered by ethanol-precipitation and dephosphorylated (30 µl reaction, 30 min, 50°C, 10 U alkaline phosphatase, Roche). The reaction was stopped by phenol/chloroform extraction and the RNA was ethanol-precipitated. The 3' adapter oligonucleotide (pUUUaaccgcatccttctcx: upper-

case, RNA; lowercase, DNA; p, phosphate; x, 4-hydroxymethylbenzyl) was then ligated to the dephosphorylated ~21 nt RNA (20 µl reaction, 30 min, 37°C, 5 µM 3' adapter, 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.2 mM ATP, 0.1 mg/ml acetylated BSA, 15% DMSO, 25 U T4 RNA ligase, Amersham-Pharmacia) (Pan and Uhlenbeck, 1992). The ligation reaction was stopped by the addition of an equal volume of 8 M urea/50 mM EDTA stopmix and directly loaded on a 15% gel. Ligation yields were greater 50%. The ligation product was recovered from the gel and 5'-phosphorylated (20 µl reaction, 30 min, 37°C, 2 mM ATP, 5 U T4 polynucleotide kinase, NEB). The phosphorylation reaction was stopped by phenol/chloroform extraction and RNA was recovered by ethanol-precipitation. Next, the 5' adapter (tactaatacgactcactAAA: uppercase, RNA; lowercase, DNA) was ligated to the phosphorylated ligation product as described above. The new ligation product was gel-purified and eluted from the gel slice in the presence of reverse transcription primer (GACTAGCTG**GAATTC**AAGGATGCGGTTAAA: bold, Eco RI site) used as carrier. Reverse transcription (15 µl reaction, 30 min, 42°C, 150 U Superscript II reverse transcriptase, Life Technologies) was followed by PCR using as 5' primer CAGCCAACG**GAATTC**ATACGACTCACTAAA (bold, Eco RI site) and the 3' RT primer. The PCR product was purified by phenol/chloroform extraction and ethanol-precipitated. The PCR product was then digested with Eco RI (NEB) and concatamerized using T4 DNA ligase (high conc., NEB). Concatamers of a size range of 200 to 800 bp were separated on a low-melt agarose gel, recovered from the gel by a standard melting and phenol extraction procedure, and ethanol-precipitated. The unpaired ends were filled in by incubation with Taq polymerase under standard conditions for 15 min at 72°C and the DNA product was directly ligated into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). Colonies were screened using PCR and M13-20 and M13 Reverse sequencing primers. PCR products were directly submitted for custom sequencing (Sequence Laboratories Göttingen GmbH, Germany). On average, four to five 21mer sequences were obtained per clone.

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1.5 2D-TLC Analysis

Nuclease P1 digestion of radiolabeled, gel-purified siRNAs and 2D-TLC was carried out as described (Zamore et al., 2000). Nuclease T2 digestion was performed in 10 µl reactions for 3 h at 50°C in 10 mM ammonium acetate (pH 4.5) using 2 µg/µl carrier tRNA and 30 U ribonuclease T2 (Life Technologies). The migration of non-radioactive standards was determined by UV shadowing. The identity of nucleoside-3',5'-disphosphates was confirmed by co-migration of the T2 digestion products with standards prepared by 5'-^{32}P-phosphorylation of commercial nucleoside 3'-monophosphates using γ-32P-ATP and T4 polynucleotide kinase (data not shown).

2. Results and Discussion

2.1 Length Requirements for Processing of dsRNA to 21 and 22 nt RNA Fragments

Lysate prepared from D. melanogaster syncytial embryos recapitulates RNAi in vitro providing a novel tool for biochemical analysis of the mechanism of RNAi (Tuschl et al., 1999; Zamore et al., 2000). In vitro and in vivo analysis of the length requirements of dsRNA for RNAi has revealed that short dsRNA (<150 bp) are less effective than longer dsRNAs in degrading target mRNA (Caplen et al., 2000; Hammond et al., 2000; Ngo et al., 1998); Tuschl et al., 1999). The reasons for reduction in mRNA degrading efficiency are not understood. We therefore examined the precise length requirement of dsRNA for target RNA degradation under optimized conditions in the Drosophila lysate (Zamore et al., 2000). Several series of dsRNAs were synthesized and directed against firefly luciferase (Pp-luc) reporter RNA. The specific suppression of target RNA expression was monitored by the dual luciferase assay (Tuschl et al., 1999) (Figures 1A and 1B). We detected specific inhibition of target RNA expression for dsRNAs as short as 38 bp, but dsRNAs of 29 to 36 bp were not effective in this process. The effect was independent of the target position and the

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degree of inhibition of Pp-luc mRNA expression correlated with the length of the dsRNA, i.e. long dsRNAs were more effective than short dsRNAs.

It has been suggested that the 21-23 nt RNA fragments generated by processing of dsRNAs are the mediators of RNA interference and co-suppression (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). We therefore analyzed the rate of 21-23 nt fragment formation for a subset of dsRNAs ranging in size between 501 to 29 bp. Formation of 21-23 nt fragments in Drosophila lysate (Figure 2) was readily detectable for 39 to 501 bp long dsRNAs but was significantly delayed for the 29 bp dsRNA. This observation is consistent with a role of 21-23 nt fragments in guiding mRNA cleavage and provides an explanation for the lack of RNAi by 30 bp dsRNAs. The length dependence of 21-23 mer formation is likely to reflect a biologically relevant control mechanism to prevent the undesired activation of RNAi by short intramolecular base-paired structures of regular cellular RNAs.

2.2 39 bp dsRNA Mediates Target RNA Cleavage at a Single Site

Addition of dsRNA and 5'-capped target RNA to the Drosophila lysate results in sequence-specific degradation of the target RNA (Tuschl et al., 1999). The target mRNA is only cleaved within the region of identity with the dsRNA and many of the target cleavage sites were separated by 21-23 nt (Zamore et al., 2000). Thus, the number of cleavage sites for a given dsRNA was expected to roughly correspond to the length of the dsRNA divided by 21. We mapped the target cleavage sites on a sense and an antisense target RNA which was 5' radiolabeled at the cap (Zamore et al., 2000) (Figures 3A and 3B). Stable 5' cleavage products were separated on a sequencing gel and the position of cleavage was determined by comparison with a partial RNase T1 and an alkaline hydrolysis ladder from the target RNA.

Consistent with the previous observation (Zamore et al., 2000), all target RNA cleavage sites were located within the region of identity to the dsRNA. The sense or the antisense traget was only cleaved once by 39 bp dsRNA. Each cleavage site was located 10 nt from the 5′ end of the region covered by the dsRNA (Figure 3B). The 52 bp dsRNA, which shares the same 5′ end with the 39 bp dsRNA, produces the same cleavage site on the sense target, located 10 nt from the 5′ end of the region of identity with the dsRNA, in addition to two weaker cleavage sites 23 and 24 nt downstream of the first site. The antisense target was only cleaved once, again 10 nt from the 5′ end of the region covered by its respective dsRNA. Mapping of the cleavage sites for the 38 to 49 bp dsRNAs shown in Figure 1 showed that the first and predominant cleavage site was always located 7 to 10 nt downstream of the region covered by the dsRNA (data not shown). This suggests that the point of target RNA cleavage is determined by the end of the dsRNA and could imply that processing to 21-23 mers starts from the ends of the duplex.

Cleavage sites on sense and antisense target for the longer 111 bp dsRNA were much more frequent than anticipated and most of them appear in clusters separated by 20 to 23 nt (Figures 3A and 3B). As for the shorter dsRNAs, the first cleavage site on the sense target is 10 nt from the 5′ end of the region spanned by the dsRNA, and the first cleavage site on the antisense target is located 9 nt from the 5′ end of region covered by the dsRNA. It is unclear what causes this disordered cleavage, but one possibility could be that longer dsRNAs may not only get processed from the ends but also internally, or there are some specificity determinants for dsRNA processing which we do not yet understand. Some irregularities to the 21-23 nt spacing were also previously noted (Zamore et al., 2000). To better understand the molecular basis of dsRNA processing and target RNA recognition, we decided to analyze the sequences of the 21-23 nt fragments generated by processing of 39, 52, and 111 bp dsRNAs in the Drosophila lysate.

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2.3 dsRNA is Processed to 21 and 22 nt RNAs by an RNase III-Like Mechanism

In order to characterize the 21-23 nt RNA fragments we examined the 5' and 3' termini of the RNA fragments. Periodate oxidation of gel-purified 21-23 nt RNAs followed by ß-elimination indicated the presence of a terminal 2' and 3' hydroxyl groups. The 21-23 mers were also responsive to alkaline phosphatase treatment indicating the presence of a 5' terminal phosphate group. The presence of 5' phosphate and 3' hydroxyl termini suggests that the dsRNA could be processed by an enzymatic activity similar to E. coli RNase III (for reviews, see (Dunn, 1982; Nicholson, 1999; Robertson, 1990; Robertson, 1982)).

Directional cloning of 21-23 nt RNA fragments was performed by ligation of a 3' and 5' adapter oligonucleotide to the purified 21-23 mers using T4 RNA ligase. The ligation products were reverse transcribed, PCR-amplified, concatamerized, cloned, and sequenced. Over 220 short RNAs were sequenced from dsRNA processing reactions of the 39, 52 and 111 bp dsRNAs (Figure 4A). We found the following length distribution: 1% 18 nt, 5% 19 nt, 12% 20 nt, 45% 21 nt, 28% 22 nt, 6% 23 nt, and 2% 24 nt. Sequence analysis of the 5' terminal nucleotide of the processed fragments indicated that oligonucleotides with a 5' guanosine were underrepresented. This bias was most likely introduced by T4 RNA ligase which discriminates against 5' phosphorylated guanosine as donor oligonucleotide; no significant sequence bias was seen at the 3' end. Many of the ~21 nt fragments derived from the 3' ends of the sense or antisense strand of the duplexes include 3' nucleotides that are derived from untemplated addition of nucleotides during RNA synthesis using T7 RNA polymerase. Interestingly, a significant number of endogenous Drosophila ~21 nt RNAs were also cloned, some of them from LTR and non-LTR retrotransposons (data not shown). This is consistent with a possible role for RNAi in transposon silencing.

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The ~21 nt RNAs appear in clustered groups (Figure 4A) which cover the entire dsRNA sequences. Apparently, the processing reaction cuts the dsRNA by leaving staggered 3' ends, another characteristic of RNase III cleavage. For the 39 bp dsRNA, two clusters of ~21 nt RNAs were found from each dsRNA-constituting strand including overhanging 3' ends, yet only one cleavage site was detected on the sense and antisense target (Figures 3A and 3B). If the ~21 nt fragments were present as single-stranded guide RNAs in a complex that mediates mRNA degradation, it could be assumed that at least two target cleavage sites exist, but this was not the case. This suggests that the ~21 nt RNAs may be present in double-stranded form in the endonuclease complex but that only one of the strands can be used for target RNA recognition and cleavage. The use of only one of the ~21 nt strands for target cleavage may simply be determined by the orientation in which the ~21 nt duplex is bound to the nuclease complex. This orientation is defined by the direction in which the original dsRNA was processed.

The ~21mer clusters for the 52 bp and 111 bp dsRNA are less well defined when compared to the 39 bp dsRNA. The clusters are spread over regions of 25 to 30 nt most likely representing several distinct subpopulations of ~21 nt duplexes and therefore guiding target cleavage at several nearby sites. These cleavage regions are still predominantly separated by 20 to 23 nt intervals. The rules determining how regular dsRNA can be processed to ~21 nt fragments are not yet understood, but it was previously observed that the approx. 21-23 nt spacing of cleavage sites could be altered by a run of uridines (Zamore et al., 2000). The specificity of dsRNA cleavage by E. coli RNase III appears to be mainly controlled by antideterminants, i.e. excluding some specific base-pairs at given positions relative to the cleavage site (Zhang and Nicholson, 1997).

To test whether sugar-, base- or cap-modification were present in processed ~21 nt RNA fragments, we incubated radiolabeled 505 bp Pp-

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luc dsRNA in lysate for 1 h, isolated the ~21 nt products, and digested it with P1 or T2 nuclease to mononucleotides. The nucleotide mixture was then analyzed by 2D thin-layer chromatography (Figure 4B). None of the four natural ribonucleotides were modified as indicated by P1 or T2 digestion. We have previously analyzed adenosine to inosine conversion in the ~21 nt fragments (after a 2 h incubation) and detected a small extent (<0.7%) deamination (Zamore et al., 2000); shorter incubation in lysate (1 h) reduced this inosine fraction to barely detectable levels. RNase T2, which cleaves 3′ of the phosphodiester linkage, produced nucleoside 3′-phosphate and nucleoside 3′,5′-diphosphate, thereby indicating the presence of a 5′-terminal monophosphate. All four nucleoside 3′,5′-diphosphates were detected and suggest that the internucleotidic linkage was cleaved with little or no sequence-specificity. In summary, the ~21 nt fragments are unmodified and were generated from dsRNA such that 5′-monophosphates and 3′-hydroxyls were present at the 5′-end.

2.4 Synthetic 21 and 22 nt RNAs Mediate Target RNA Cleavage

Analysis of the products of dsRNA processing indicated that the ~21 nt fragments are generated by a reaction with all the characteristics of an RNase III cleavage reaction (Dunn, 1982; Nicholson, 1999; Robertson, 1990; Robertson, 1982). RNase III makes two staggered cuts in both strands of the dsRNA, leaving a 3′ overhang of about 2 nt. We chemically synthesized 21 and 22 nt RNAs, identical in sequence to some of the cloned ~21 nt fragments, and tested them for their ability to mediate target RNA degradation (Figures 5A and 5B). The 21 and 22 nt RNA duplexes were incubated at 100 nM concentrations in the lysate, a 10-fold higher concentrations than the 52 bp control dsRNA. Under these conditions, target RNA cleavage is readily detectable. Reducing the concentration of 21 and 22 nt duplexes from 100 to 10 nM does still cause target RNA cleavage. Increasing the duplex concentration from 100 nM to 1000 nM however does not further increase target cleavage, probably due to a limiting protein factor within the lysate.

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In contrast to 29 or 30 bp dsRNAs that did not mediate RNAi, the 21 and 22 nt dsRNAs with overhanging 3' ends of 2 to 4 nt mediated efficient degradation of target RNA (duplexes 1, 3, 4, 6, Figures 5A and 5B). Blunt-ended 21 or 22 nt dsRNAs (duplexes 2, 5, and 7, Figures 5A and 5B) were reduced in their ability to degrade the target and indicate that overhanging 3' ends are critical for reconstitution of the RNA-protein nuclease complex. The single-stranded overhangs may be required for high affinity binding of the ~ 21 nt duplex to the protein components. A 5' terminal phosphate, although present after dsRNA processing, was not required to mediate target RNA cleavage and was absent from the short synthetic RNAs.

The synthetic 21 and 22 nt duplexes guided cleavage of sense as well as antisense targets within the region covered by the short duplex. This is an important result considering that a 39 bp dsRNA, which forms two pairs of clusters of ~21 nt fragments (Fig. 2), cleaved sense or antisense target only once and not twice. We interpret this result by suggesting that only one of two strands present in the ~21 nt duplex is able to guide target RNA cleavage and that the orientation of the ~21 nt duplex in the nuclease complex is determined by the initial direction of dsRNA processing. The presentation of an already perfectly processed ~21 nt duplex to the in vitro system however does allow formation of the active sequence-specific nuclease complex with two possible orientations of the symmetric RNA duplex. This results in cleavage of sense as well as antisense target within the region of identity with the 21 nt RNA duplex.

The target cleavage site is located 11 or 12 nt downstream of the first nucleotide that is complementary to the 21 or 22 nt guide sequence, i.e. the cleavage site is near center of the region covered by the 21 or 22 nt RNAs (Figures 4A and 4B). Displacing the sense strand of a 22 nt duplex by two nucleotides (compare duplexes 1 and 3 in Figure 5A) displaced the cleavage site of only the antisense target by two nucleotides. Displacing both sense and antisense strand by two nucleotides shifted both cleavage

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sites by two nucleotides (compare duplexes 1 and 4). We predict that it will be possible to design a pair of 21 or 22 nt RNAs to cleave a target RNA at almost any given position.

The specificity of target RNA cleavage guided by 21 and 22 nt RNAs appears exquisite as no aberrant cleavage sites are detected (Figure 5B). It should however be noted, that the nucleotides present in the 3' overhang of the 21 and 22 nt RNA duplex may contribute less to substrate recognition than the nucleotides near the cleavage site. This is based on the observation that the 3' most nucleotide in the 3' overhang of the active duplexes 1 or 3 (Figure 5A) is not complementary to the target. A detailed analysis of the specificity of RNAi can now be readily undertaken using synthetic 21 and 22 nt RNAs.

Based on the evidence that synthetic 21 and 22 nt RNAs with overhanging 3' ends mediate RNA interference, we propose to name the ~21 nt RNAs "short interfering RNAs" or siRNAs and the respective RNA-protein complex a "small interfering ribonucleoprotein particle" or siRNP.

2.5 3' Overhangs of 20 nt on short dsRNAs inhibit RNAi

We have shown that short blunt-ended dsRNAs appear to be processed from the ends of the dsRNA. During our study of the length dependence of dsRNA in RNAi, we have also analyzed dsRNAs with 17 to 20 nt overhanging 3' ends and found to our surprise that they were less potent than blunt-ended dsRNAs. The inhibitory effect of long 3' ends was particularly pronounced for dsRNAs up to 100 bp but was less dramatic for longer dsRNAs. The effect was not due to imperfect dsRNA formation based on native gel analysis (data not shown). We tested if the inhibitory effect of long overhanging 3' ends could be used as a tool to direct dsRNA processing to only one of the two ends of a short RNA duplex.

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We synthesized four combinations of the 52 bp model dsRNA, blunt-ended, 3′ extension on only the sense strand, 3′-extension on only the antisense strand, and double 3′ extension on both strands, and mapped the target RNA cleavage sites after incubation in lysate (Figures 6A and 6B). The first and predominant cleavage site of the sense target was lost when the 3′ end of the antisense strand of the duplex was extended, and vice versa, the strong cleavage site of the antisense target was lost when the 3′ end of sense strand of the duplex was extended. 3′ Extensions on both strands rendered the 52 bp dsRNA virtually inactive. One explanation for the dsRNA inactivation by ~20 nt 3′ extensions could be the association of single-stranded RNA-binding proteins which could interfere with the association of one of the dsRNA-processing factors at this end. This result is also consistent with our model where only one of the strands of the siRNA duplex in the assembled siRNP is able to guide target RNA cleavage. The orientation of the strand that guides RNA cleavage is defined by the direction of the dsRNA processing reaction. It is likely that the presence of 3′ staggered ends may facilitate the assembly of the processing complex. A block at the 3′ end of the sense strand will only permit dsRNA processing from the opposing 3′ end of the antisense strand. This in turn generates siRNP complexes in which only the antisense strand of the siRNA duplex is able to guide sense target RNA cleavage. The same is true for the reciprocal situation.

The less pronounced inhibitory effect of long 3′ extensions in the case of longer dsRNAs (≥500 bp, data not shown) suggests to us that long dsRNAs may also contain internal dsRNA-processing signals or may get processed cooperatively due to the association of multiple cleavage factors.

2.6 A Model for dsRNA-Directed mRNA Cleavage

The new biochemical data update the model for how dsRNA targets mRNA for destruction (Figure 7). Double-stranded RNA is first processed to short

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RNA duplexes of predominantly 21 and 22 nt in length and with staggered 3' ends similar to an RNase III-like reaction (Dunn, 1982; Nicholson, 1999; Robertson, 1982). Based on the 21-23 nt length of the processed RNA fragments it has already been speculated that an RNase III-like activity may be involved in RNAi (Bass, 2000). This hypothesis is further supported by the presence of 5' phosphates and 3' hydroxyls at the termini of the siRNAs as observed in RNase III reaction products (Dunn, 1982; Nicholson, 1999). Bacterial RNase III and the eukaryotic homologs Rnt1p in S. cerevisiae and Pac1p in S. pombe have been shown to function in processing of ribosomal RNA as well as snRNA and snoRNAs (see for example Chanfreau et al., 2000).

Little is known about the biochemistry of RNase III homologs from plants, animals or human. Two families of RNase III enzymes have been identified predominantly by database-guided sequence analysis or cloning of cDNAs. The first RNase III family is represented by the 1327 amino acid long D. melanogaster protein drosha (Acc. AF116572). The C-terminus is composed of two RNase III and one dsRNA-binding domain and the N-terminus is of unknown function. Close homologs are also found in C. elegans (Acc. AF160248) and human (Acc. AF189011) (Filippov et al., 2000; Wu et al., 2000). The drosha-like human RNase III was recently cloned and characterized (Wu et al., 2000). The gene is ubiquitously expressed in human tissues and cell lines, and the protein is localized in the nucleus and the nucleolus of the cell. Based on results inferred from antisense inhibition studies, a role of this protein for rRNA processing was suggested. The second class is represented by the C. elegans gene K12H4.8 (Acc. S44849) coding for a 1822 amino acid long protein. This protein has an N-terminal RNA helicase motif which is followed by 2 RNase III catalytic domains and a dsRNA-binding motif, similar to the drosha RNase III family. There are close homologs in S. pombe (Acc. Q09884), A. thaliana (Acc. AF187317), D. melanogaster (Acc. AE003740), and human (Acc. AB028449) (Filippov et al., 2000; Jacobsen et al., 1999; Matsuda et al.,

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2000). Possibly the K12H4.8 RNase III/helicase is the likely candidate to be involved in RNAi.

Genetic screens in C. elegans identified rde-1 and rde-4 as essential for activation of RNAi without an effect on transposon mobilization or co-suppression (Dernburg et al., 2000; Grishok et al., 2000; Ketting and Plasterk, 2000; Tabara et al., 1999). This led to the hypothesis that these genes are important for dsRNA processing but are not involved in mRNA target degradation. The function of both genes is as yet unknown, the rde-1 gene product is a member of a family of proteins similar to the rabbit protein eIF2C (Tabara et al., 1999), and the sequence of rde-4 has not yet been described. Future biochemical characterization of these proteins should reveal their molecular function.

Processing to the siRNA duplexes appears to start from the ends of both blunt-ended dsRNAs or dsRNAs with short (1-5 nt) 3′ overhangs, and proceeds in approximately 21-23 nt steps. Long (~20 nt) 3′ staggered ends on short dsRNAs suppress RNAi, possibly through interaction with single-stranded RNA-binding proteins. The suppression of RNAi by single-stranded regions flanking short dsRNA and the lack of siRNA formation from short 30 bp dsRNAs may explain why structured regions frequently encountered in mRNAs do not lead to activation of RNAi.

Without wishing to be bound by theory, we presume that the dsRNA-processing proteins or a subset of these remain associated with the siRNA duplex after the processing reaction. The orientation of the siRNA duplex relative to these proteins determines which of the two complementary strands functions in guiding target RNA degradation. Chemically synthesized siRNA duplexes guide cleavage of sense as well as antisense target RNA as they are able to associate with the protein components in either of the two possible orientation.

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The remarkable finding that synthetic 21 and 22 nt siRNA duplexes can be used for efficient mRNA degradation provides new tools for sequence-specific regulation of gene expression in functional genomics as well as biomedical studies. The siRNAs may be effective in mammalian systems where long dsRNAs cannot be used due to the activation of the PKR response (Clemens, 1997). As such, the siRNA duplexes represent a new alternative to antisense or ribozyme therapeutics.

Example 2

RNA Interference in Human Tissue Cultures

1. **Methods**

1.1 RNA preparation

21 nt RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified (Example 1), followed by Sep-Pak C18 cartridge (Waters, Milford, MA, USA) purification (Tuschl, 1993). The siRNA sequences targeting GL2 (Acc. X65324) and GL3 luciferase (Acc. U47296) corresponded to the coding regions 153-173 relative to the first nucleotide of the start codon, siRNAs targeting RL (Acc. AF025846) corresponded to region 119-129 after the start codon. Longer RNAs were transcribed with T7 RNA polymerase from PCR products, followed by gel and Sep-Pak purification. The 49 and 484 bp GL2 or GL3 dsRNAs corresponded to position 113-161 and 113-596, respectively, relative to the start of translation; the 50 and 501 bp RL dsRNAs corresponded to position 118-167 and 118-618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (Kehlenbach, 1998), whereby 50 and 501 bp hG dsRNA corresponded to position 118-167 and 118-618, respectively, to the start codon.

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For annealing of siRNAs, 20 μM single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h at 37°C. The 37°C incubation step was extended overnight for the 50 and 500 bp dsRNAs and these annealing reactions were performed at 8.4 μM and 0.84 μM strand concentrations, respectively.

1.2 Cell Culture

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 25°C. 293, NIH/3T3, HeLa S3, COS-7 cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were regularly passaged to maintain exponential growth. 24 h before transfection at approx. 80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1-3 x 10^5 cells/ml) and transferred to 24-well plates (500 μl/well). S2 cells were not trypsinized before splitting. Transfection was carried out with Lipofectamine 2000 reagent (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 μg pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 μg pRL-TK (Promega) and 0.28 μg siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 μl per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cell lines after co-transfection of 1.1 μg hGFP-encoding pAD3 and 0.28 μg invGL2 inGL2 siRNA and were 70-90%. Reporter plasmids were amplified in XL-1 Blue (Stratagene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

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2. Results

To test whether siRNAs are also capable of mediating RNAi in tissue culture, we synthesized 21 nt siRNA duplexes with symmetric 2 nt 3' overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases (Fig. 8a, b). The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL into *D. melanogaster Schneider S2* cells or mammalian cells using cationic liposomes. Luciferase activities were determined 20 h after transfection. In all cell lines tested, we observed specific reduction of the expression of the reporter genes in the presence of cognate siRNA duplexes (Fig. 9a-j). Remarkably, the absolute luciferase expression levels were unaffected by non-cognate siRNAs, indicating the absence of harmful side effects by 21 nt RNA duplexes (e.g. Fig. 10a-d for HeLa cells). In *D. melanogaster* S2 cells (Fig. 9a, b), the specific inhibition of luciferases was complete. In mammalian cells, where the reporter genes were 50- to 100-fold stronger expressed, the specific suppression was less complete (Fig. 9c-j). GL2 expression was reduced 3- to 12-fold, GL3 expression 9- to 25-fold and RL expression 1- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (Fig. 9i, j). The lack of reduction of RL expression in 293 cells may be due to its 5- to 20-fold higher expression compared to any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

The 2 nt 3' overhang in all siRNA duplexes, except for uGL2, was composed of (2'-deoxy) thymidine. Substituion of uridine by thymidine in the 3' overhang was well tolerated in the *D. melanogaster* in vitro sytem and the sequence of the overhang was uncritical for target recognition. The thymidine overhang was chosen, because it is supposed to enhance nuclease

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resistance of siRNAs in the tissue culture medium and within transfected cells. Indeed, the thymidine-modified GL2 siRNA was slightly more potent than the unmodified uGL2 siRNA in all cell lines tested (Fig. 9a, c, e, g, i). It is conceivable that further modifications of the 3' overhanging nucleotides may provide additional benefits to the delivery and stability of siRNA duplexes.

In co-transfection experiments, 25 nM siRNA duplexes with respect to the final volume of tissue culture medium were used (Fig. 9, 10). Increasing the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies due to competition for liposome encapsulation between plasmid DNA and siRNA (data not shown). Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect (data not shown), even though the siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene targeting experiments.

In order to monitor the effect of longer dsRNAs on mammalian cells, 50 and 500 bp dsRNAs cognate to the reporter genes were prepared. As non-specific control, dsRNAs from humanized GFP (hG) (Kehlenbach, 1998) was used. When dsRNAs were co-transfected, in identical amounts (not concentrations) to the siRNA duplexes, the reporter gene expression was strongly and unspecifically reduced. This effect is illustrated for HeLa cells as a representative example (Fig. 10a-d). The absolute luciferase activities were decreased unspecifically 10- to 20-fold by 50 bp dsRNA and 20- to 200-fold by 500 bp dsRNA co-transfection, respectively. Similar unspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold unspecific reduction was observed only for 500 bp dsRNAs. Un-

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specific reduction in reporter gene expression by dsRNA > 30 bp was expected as part of the interferon response.

Surprisingly, despite the strong unspecific decrease in reporter gene expression, we reproducibly detected additional sequence-specific, dsRNA-mediated silencing. The specific silencing effects, however, were only apparent when the relative reporter gene activities were normalized to the hG dsRNA controls (Fig. 10e, f). A 2- to 10-fold specific reduction in response to cognate dsRNA was observed, also in the other three mammalian cell lines tested (data not shown). Specific silencing effects with dsRNAs (356-1662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments (Ui-Tei, 2000). Also CHO-K1 cells appear to be deficient in the interferon response. In another report, 293, NIH/3T3 and BHK-21 cells were tested for RNAi using luciferase/lacZ reporter combinations and 829 bp specific lacZ or 717 bp unspecific GFP dsRNA (Caplen, 2000). The failure of detecting RNAi in this case may be due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, our results indicate that RNAi is active in mammalian cells, but that the silencing effect is difficult to detect, if the interferon system is activated by dsRNA > 30 bp.

In summary, we have demonstrated for the first time siRNA-mediated gene silencing in mammalian cells. The use of short siRNAs holds great promise for inactivation of gene function in human tissue culture and the development of gene-specific therapeutics.

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EXHIBIT 9

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Claims

1. Isolated double-stranded RNA molecule, wherein each RNA strand has a length from 19-23 nucleotides, wherein said RNA molecule is capable of target-specific nucleic acid modifications.

2. The RNA molecule of claim 1 wherein at least one strand has a 3'-overhang from 1-5 nucleotides.

3. The RNA molecule of claim 1 or 2 capable of target-specific RNA interference and/or DNA methylation.

4. The RNA molecule of any one of claims 1-3, wherein each strand has a length from 20-22 nucleotides.

5. The RNA molecule of any one of claims 2-4, wherein the 3'-overhang is from 1-3 nucleotides.

6. The RNA molecule of any one of claims 2-5, wherein the 3'-overhang is stabilized against degradation.

7. The RNA molecule of any one of claims 1-6, which contains at least one modified nucleotide analogue.

8. The RNA molecule of claim 7, wherein the modified nucleotide analogue is selected from sugar- or backbone modified ribonucleotides.

9. The RNA molecule according to claim 7 or 8, wherein the nucleotide analogue is a sugar-modified ribonucleotide, wherein the 2'-OH group is replaced by a group selected from H, OR, R, halo, SH, SR^1,

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NH_2, NHR, NR_2 or CN, wherein R is C_1-C_8 alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I.

10. The RNA molecule of claim 7 or 8, wherein the nucleotide analogue is a backbone-modified ribonucleotide containing a phosphothioate group.

11. The RNA molecule of any one of claims 1-10, which has a sequence having an identity of at least 50 percent to a predetermined mRNA target molecule.

12. The RNA molecule of claim 11, wherein the identity is at least 70 percent.

13. A method of preparing a double-stranded RNA molecule of any one of claims 1-12 comprising the steps:

(a) synthesizing two RNA strands each having a length from 19-23 nucleotides, wherein said RNA strands are capable of forming a double-stranded RNA molecule,

(b) combining the synthesized RNA strands under conditions, wherein a double-stranded RNA molecule is formed, which is capable of target-specific nucleic acid modifications.

14. The method of claim 13, wherein the RNA strands are chemically synthesized.

15. The method of claim 13, wherein the RNA strands are enzymatically synthesized.

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16. A method of mediating target-specific nucleic acid modifications in a cell or an organism comprising the steps:

(a) contacting said cell or organism with the double-stranded RNA molecule of any one of claims 1-12 and

(b) mediating a target-specific nucleic acid modification effected by the double-stranded RNA towards a target nucleic acid having a sequence portion substantially corresponding to the double-stranded RNA.

17. The method of claim 16, wherein the nucleic acid modification is RNA interference and/or DNA methylation.

18. Use of the method of claim 16 or 17 for determining the function of a gene in a cell or an organism.

19. Use of the method of claim 16 or 17 for modulating the function of a gene in a cell or an organism.

20. The use of claim 16 or 17, wherein the gene is associated with a pathological condition.

21. The use of claim 20, wherein the gene is a pathogen-associated gene.

22. The use of claim 21, wherein the gene is a viral gene.

23. The use of claim 20, wherein the gene is a tumor-associated gene.

24. The use of claim 20, wherein the gene is an autoimmune disease-associated gene.

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25. Pharmaceutical composition containing as an active agent at least one double-stranded RNA molecule of any one of claims 1-12 and a pharmaceutical carrier.

26. The composition of claim 25 for diagnostic applications.

27. The composition of claim 25 for therapeutic applications.

T00320 T59v2009

Abstract

Double-stranded RNA (dsRNA) induces sequence-specific post-transcriptional gene silencing in many organisms by a process known as RNA interference (RNAi). Using a Drosophila in vitro system, we demonstrate that 19-23 nt short RNA fragments are the sequence-specific mediators of RNAi. The short interfering RNAs (siRNAs) are generated by an RNase III-like processing reaction from long dsRNA. Chemically synthesized siRNA duplexes with overhanging 3' ends mediate efficient target RNA cleavage in the lysate, and the cleavage site is located near the center of the region spanned by the guiding siRNA. Furthermore, we provide evidence that the direction of dsRNA processing determines whether sense or antisense target RNA can be cleaved by the produced siRNP complex.

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EXHIBIT 1005

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FIGURE 1A

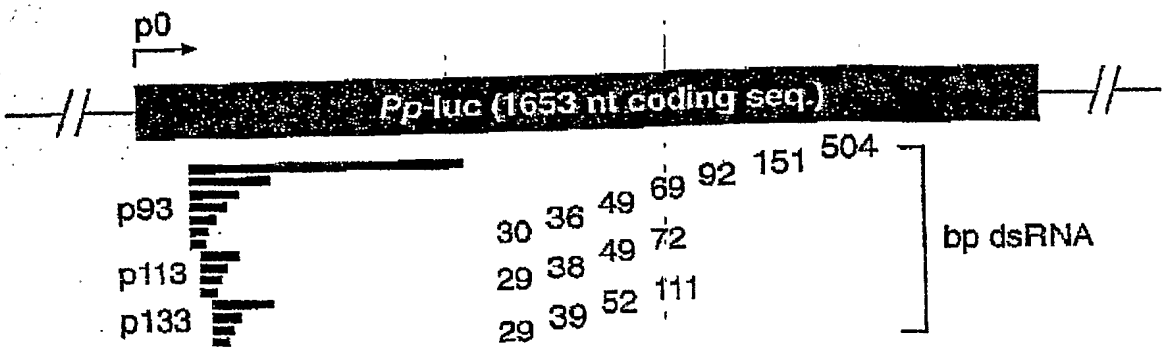


FIGURE 1B

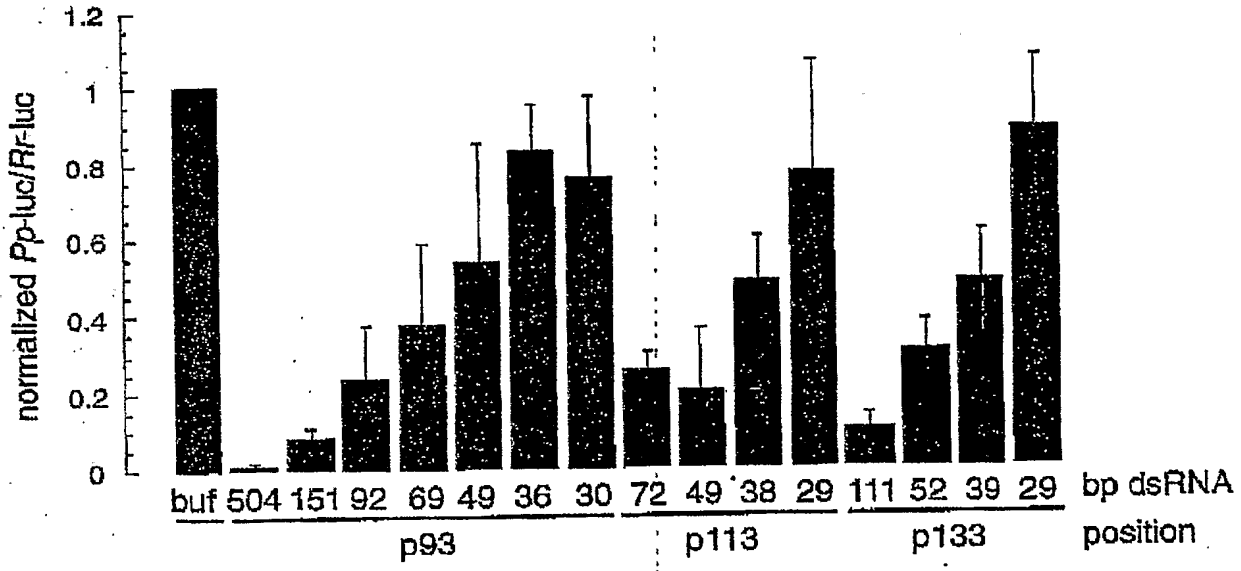
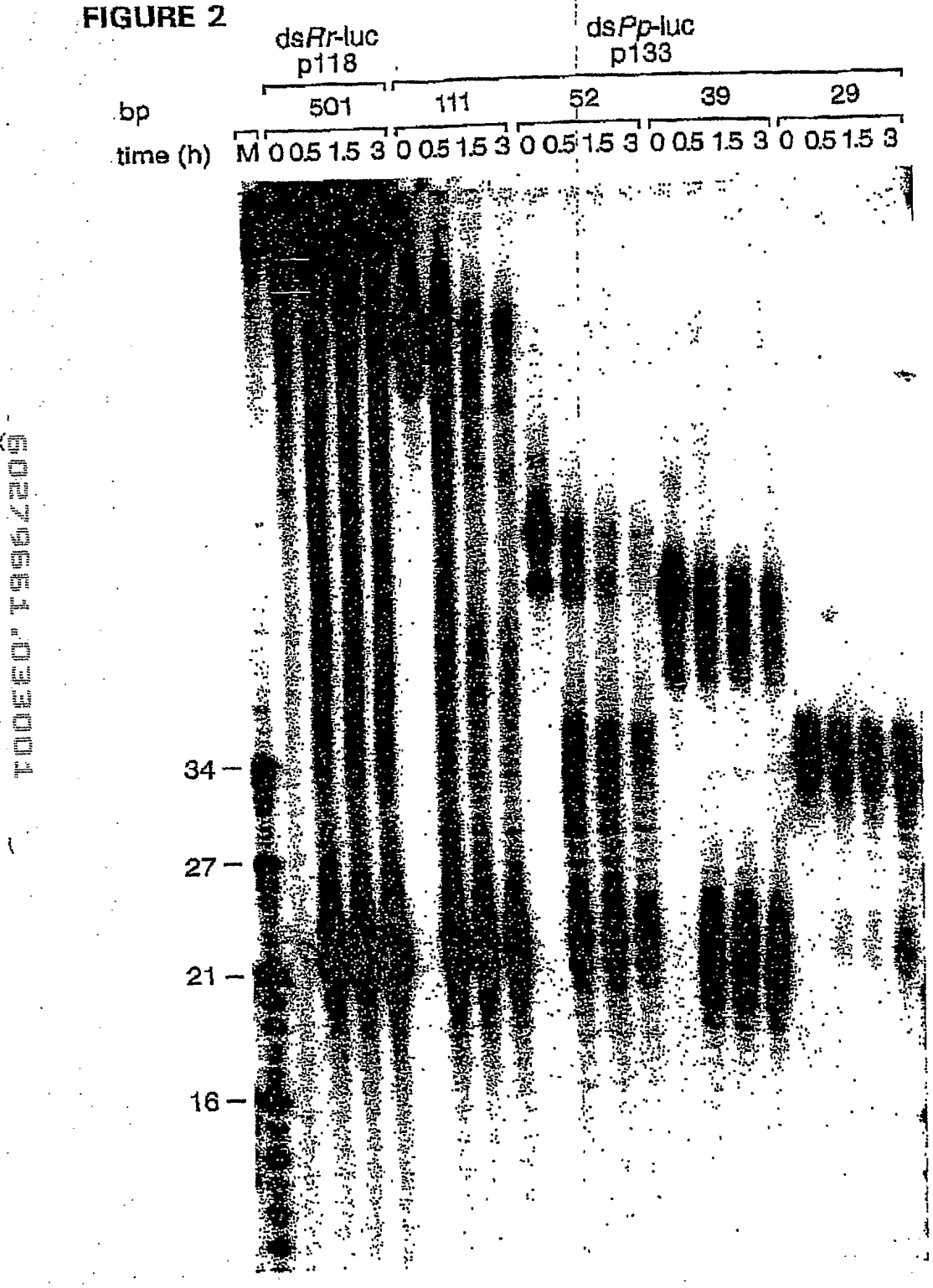
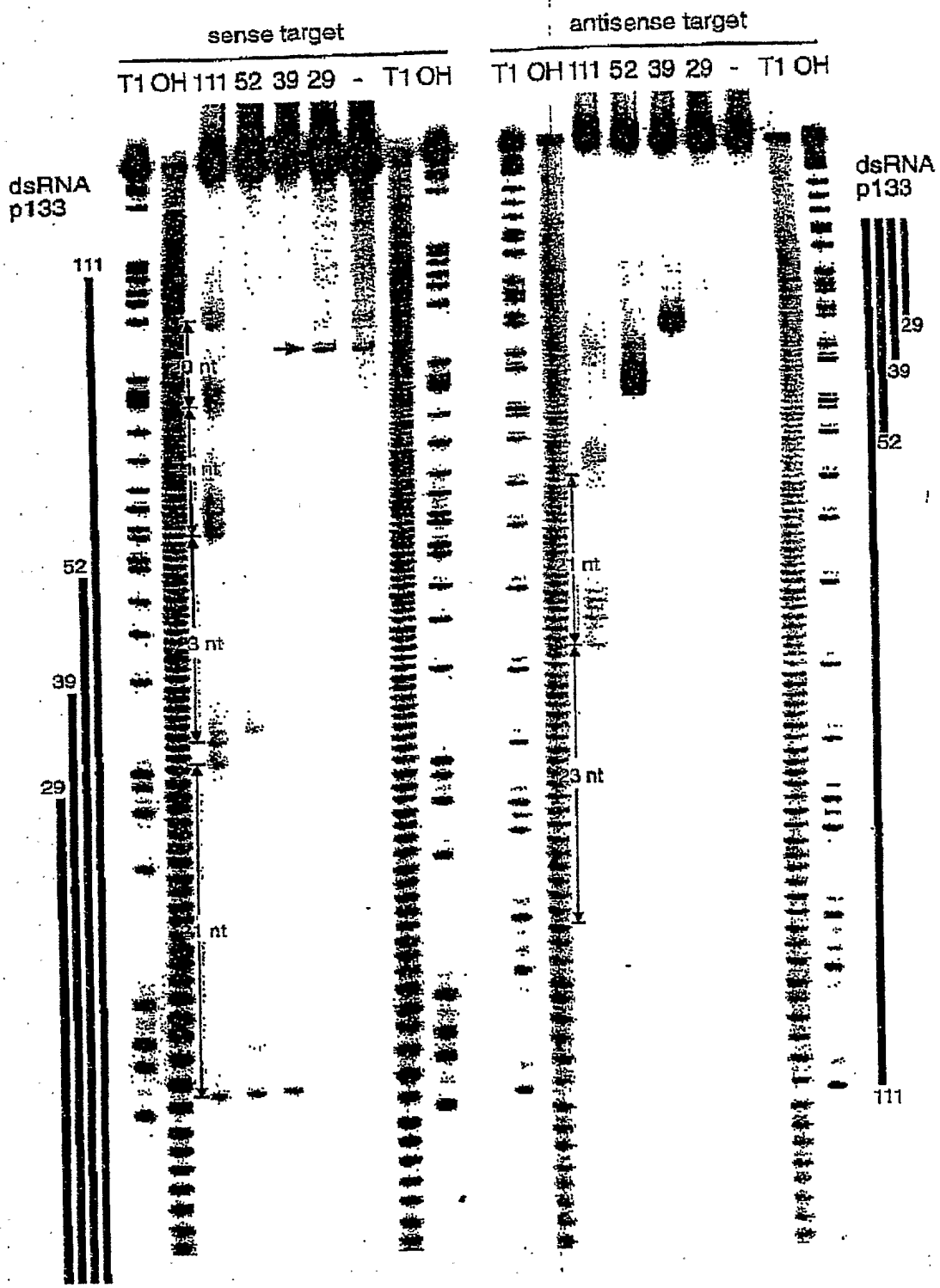


FIGURE 2


ds*Rr*-luc p118
ds*Pp*-luc p133
bp
501
111
52
39
29
time (h)
M 0 0.5 1.5 3 0 0.5 1.5 3 0 0.5 1.5 3 0 0.5 1.5 3 0 0.5 1.5 3
34
27
21
16

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FIGURE 3A



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FIGURE 3B

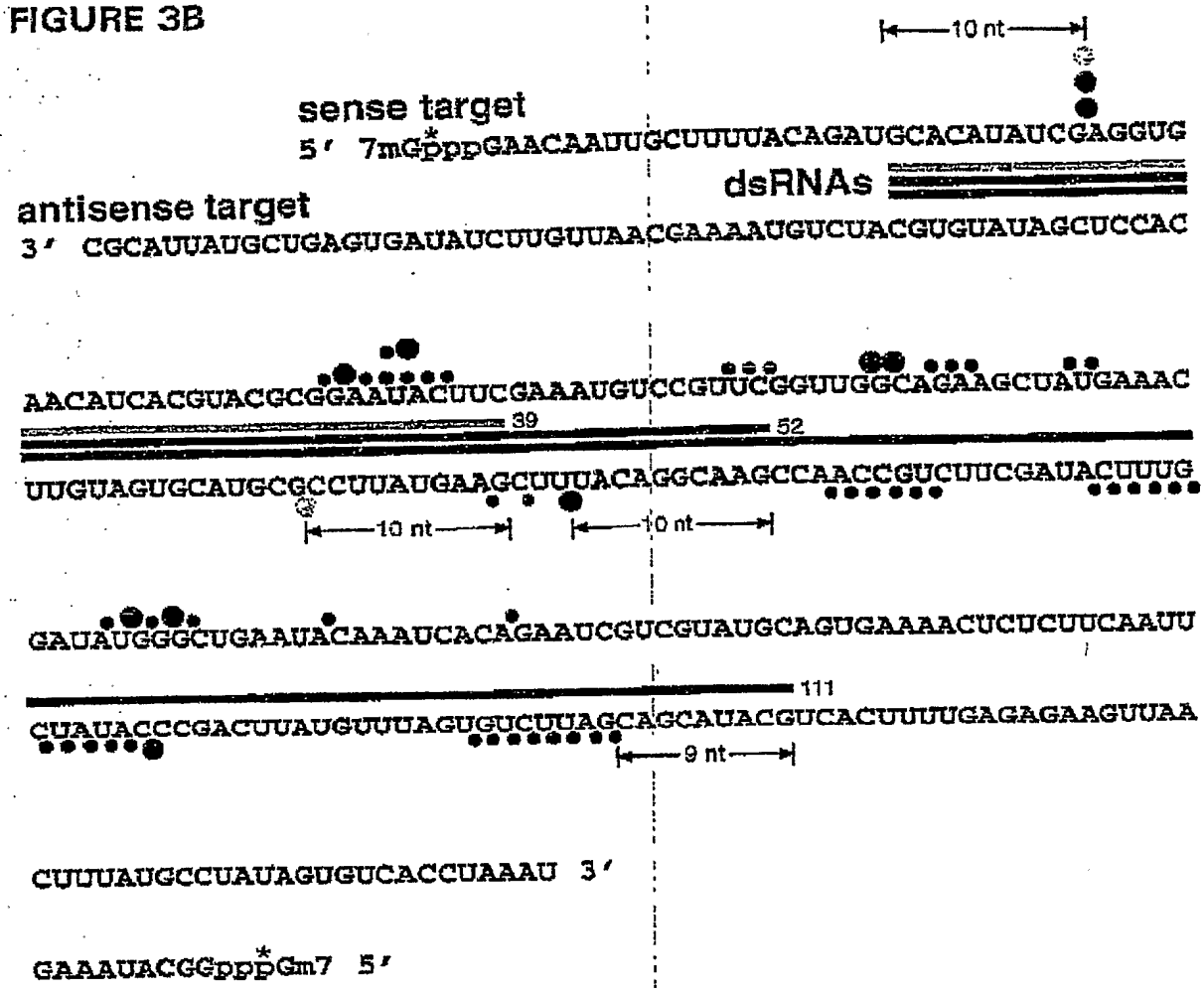
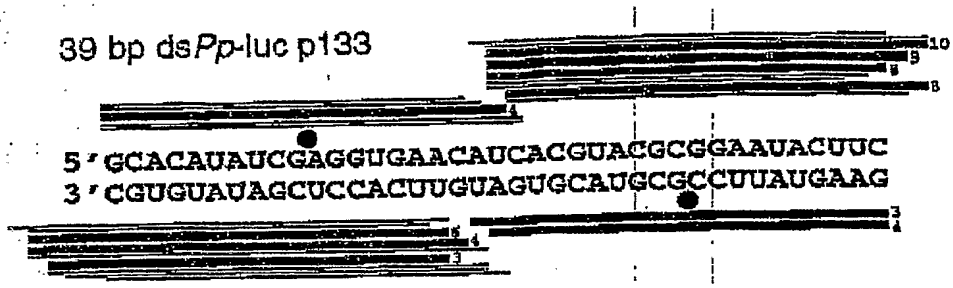
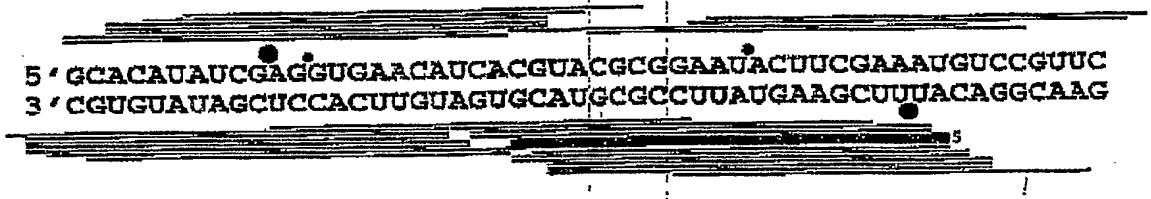


FIGURE 4A

39 bp ds*Pp*-luc p133



52 bp ds*Pp*-luc p133



111 bp ds*Pp*-luc p133



FIGURE 4B

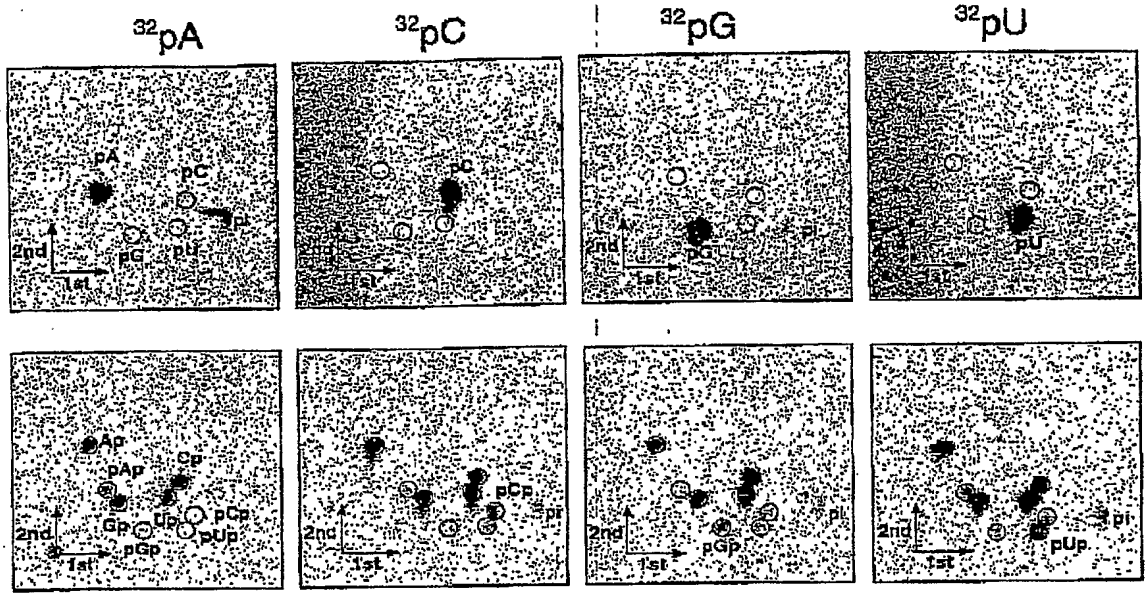
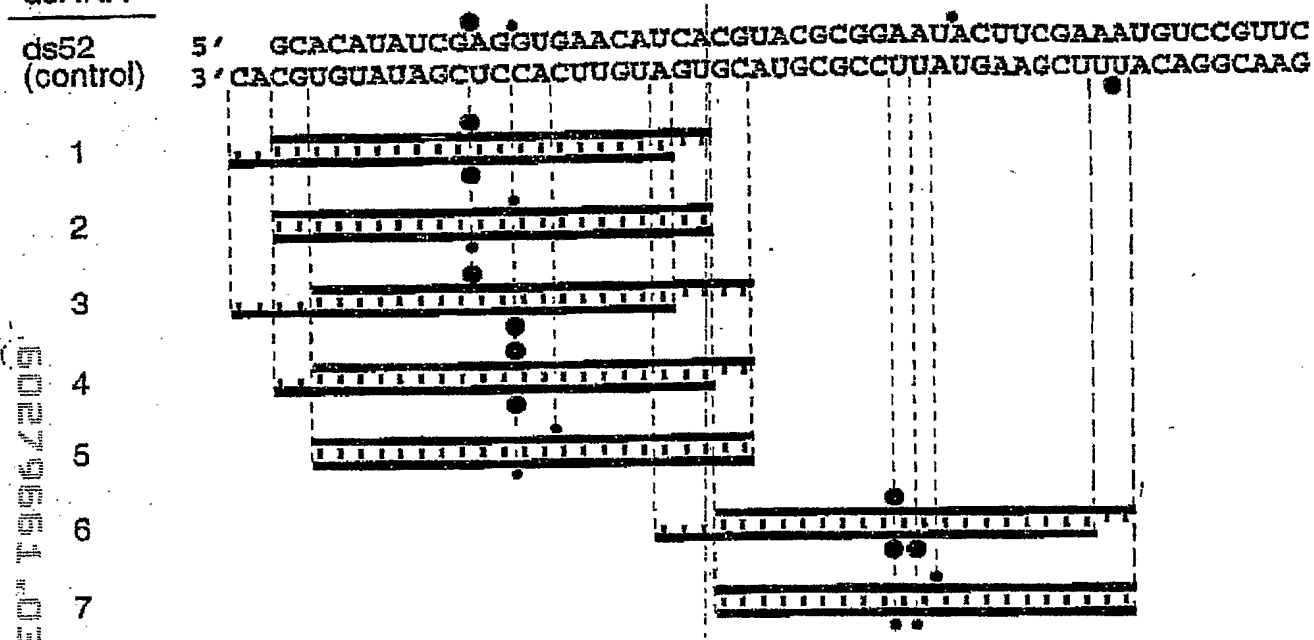


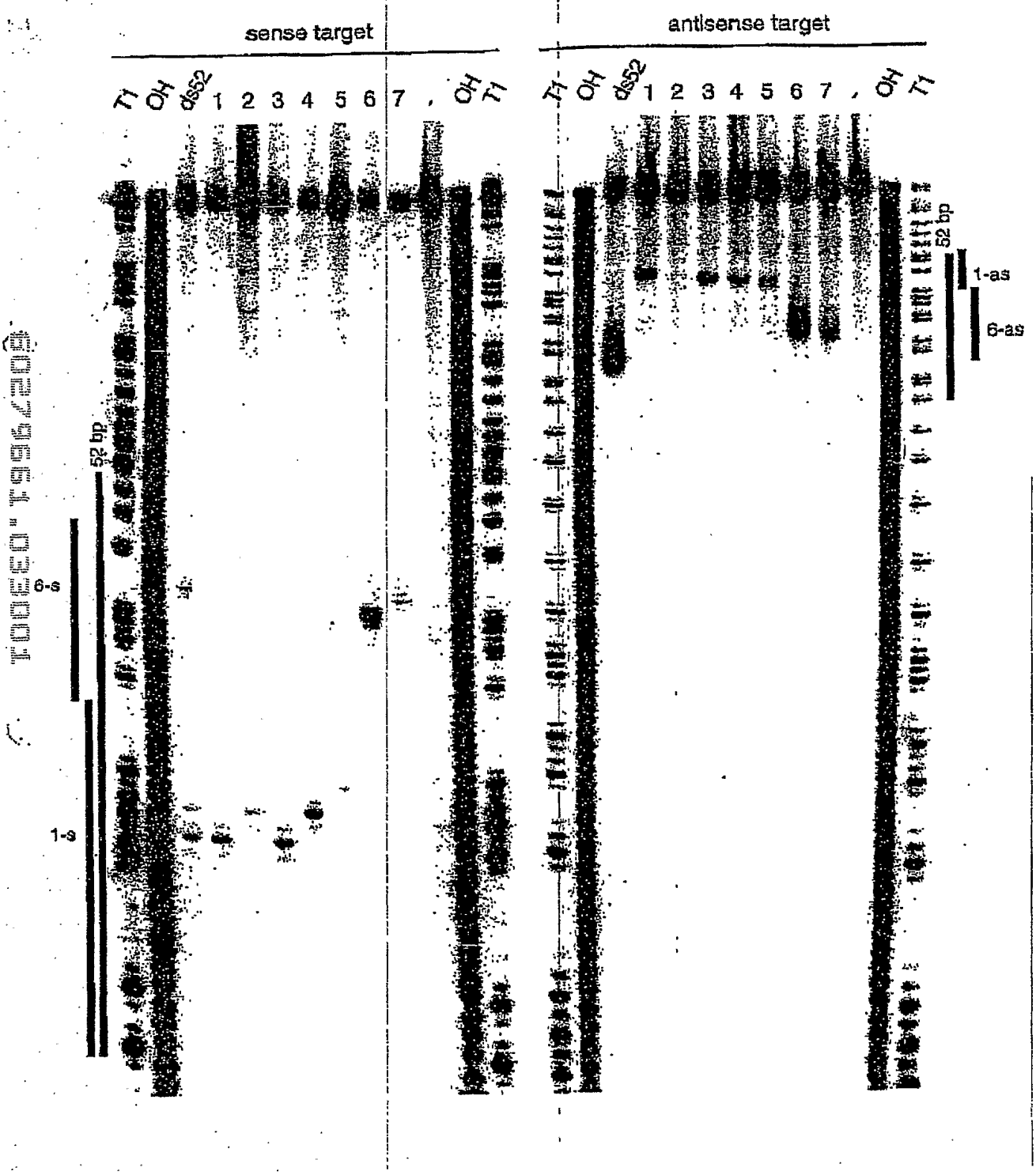
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FIGURE 5A



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FIGURE 5B


sense target
antisense target
T1
OH
dss2
1
2
3
4
5
6
7
52 bp
6-s
1-s
1-as
6-as

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FIGURE 6A

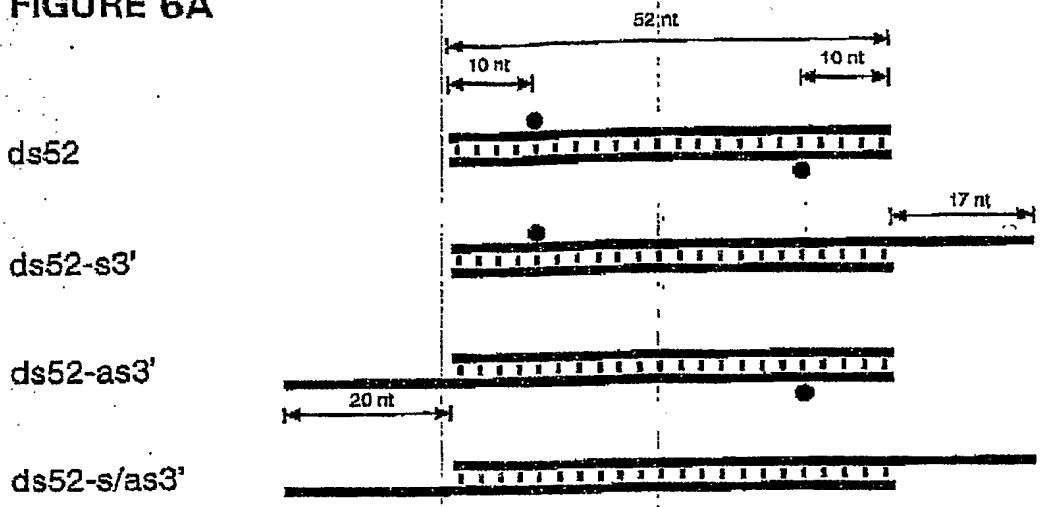
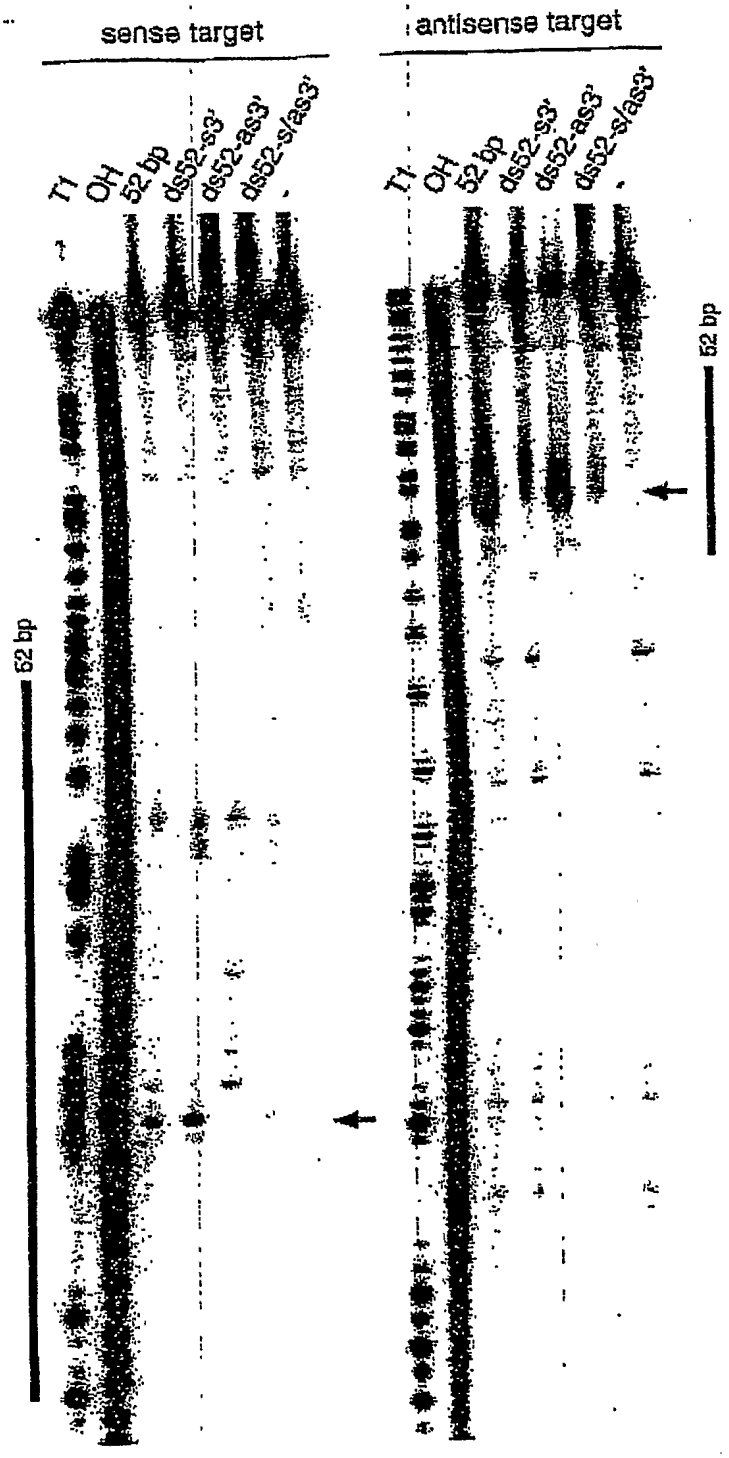
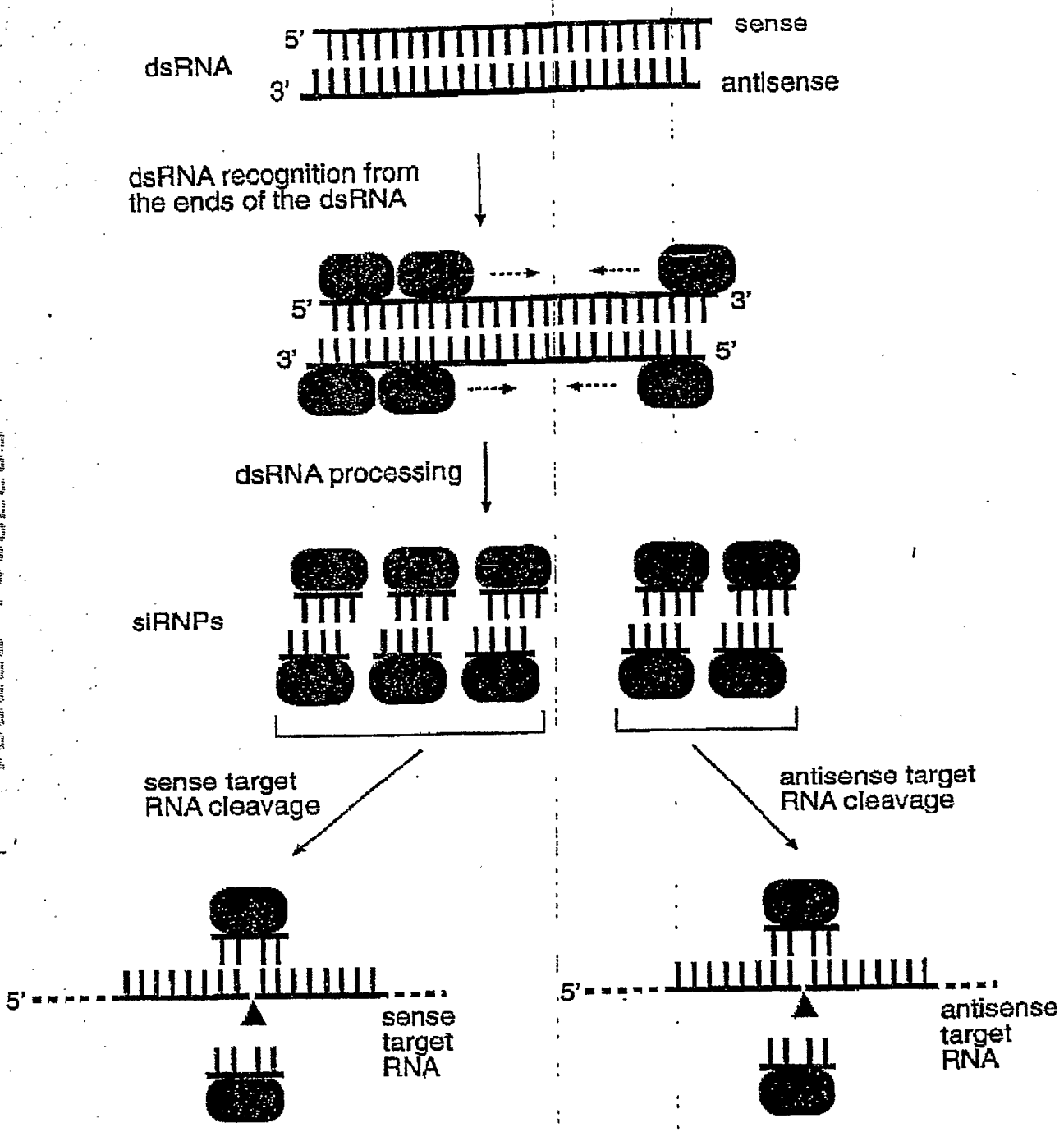


FIGURE 6B


sense target
antisense target
T1
OH
52 bp
ds52-s3'
ds52-as3'
ds52-s/as3'
62 bp

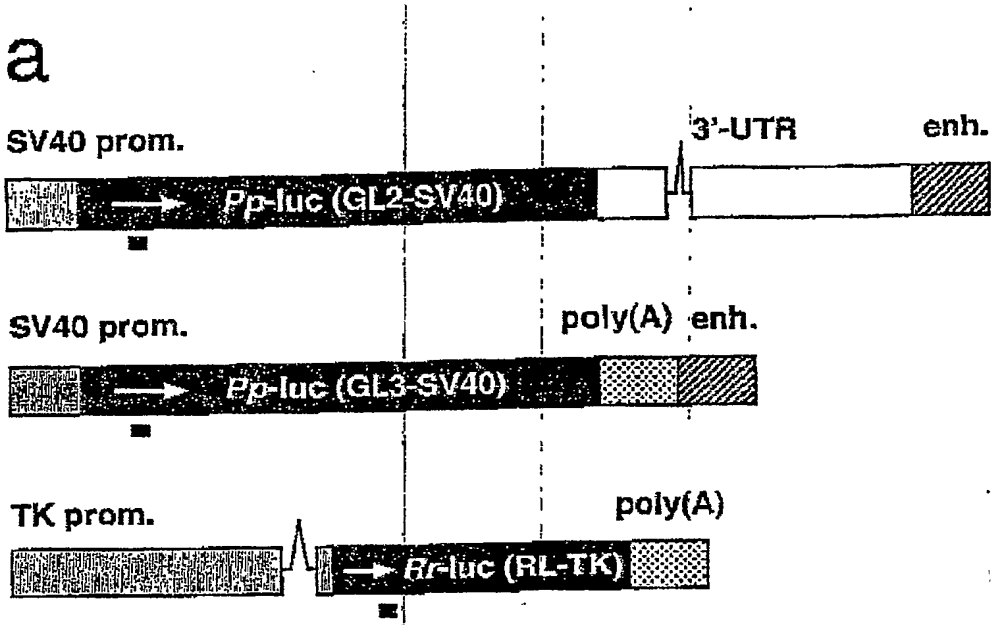
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FIGURE 7



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FIGURE 8



b

siRNA duplex	
uGL2	5′ CGUACGCGGAAUACUUCGAUU UUGCAUGCGCCUUAUGAAGCU 5′
GL2	5′ CGUACGCGGAAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5′
GL3	5′ CUUACGCUGAGUACUUCGATT TTGAAUGCGACUCAUGAAGCU 5′
invGL2	5′ AGCUUCAUAAGGCGCAUGCTT TTUCGAAGUAUUCCGCGUACG 5′
RL	5′ AAACAUGCAGAAAAUGCUGTT TTUUUGUACGUCUUUUACGAC 5′

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FIGURE 9

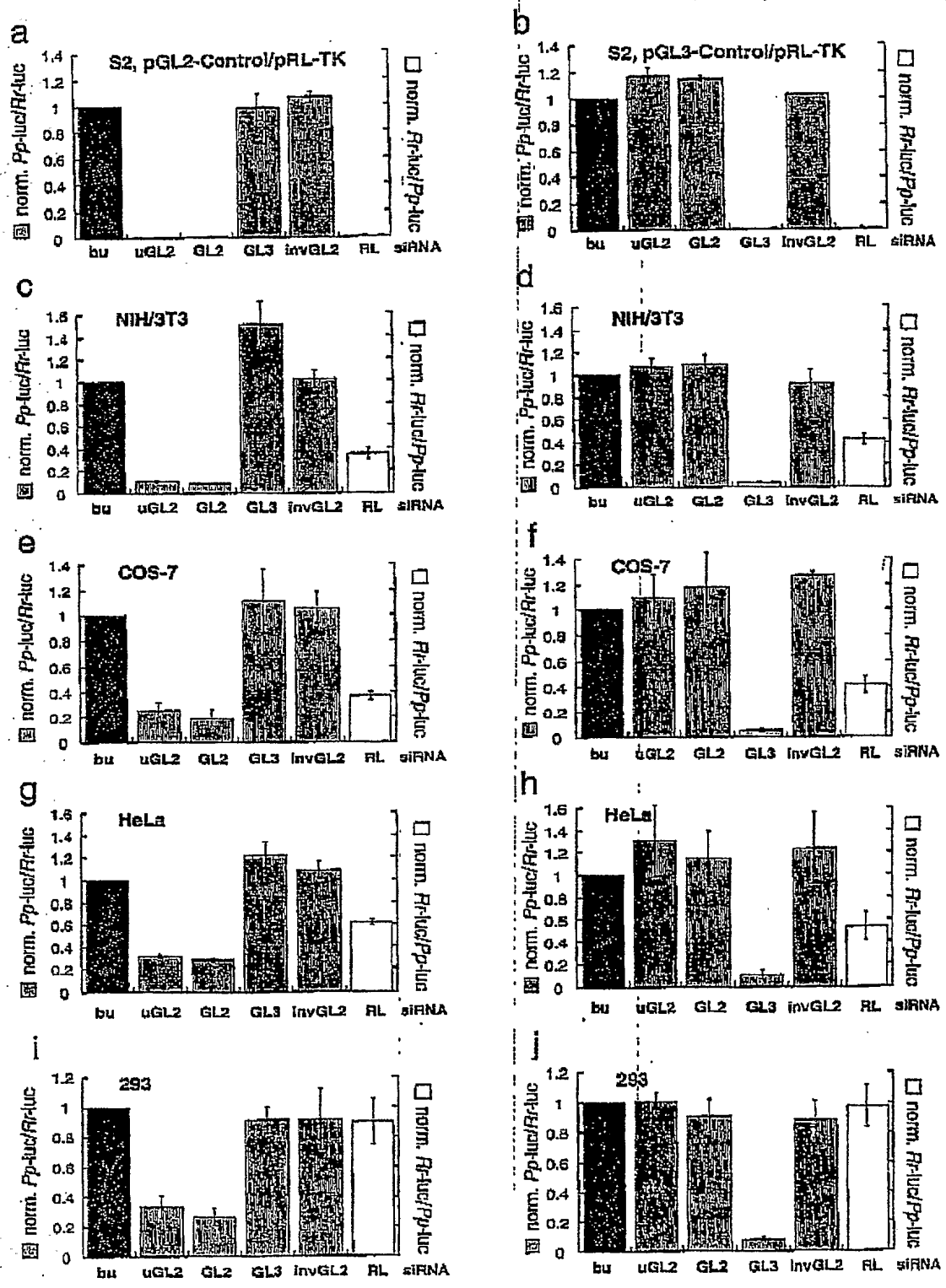

a
S2, pGL2-Control/pRL-TK
b
S2, pGL3-Control/pRL-TK
c
NIH/3T3
d
NIH/3T3
e
COS-7
f
COS-7
g
HeLa
h
HeLa
i
293
j
293
norm. Pp-luc/Rr-luc
norm. Rr-luc/Pp-luc
bu
uGL2
GL2
GL3
invGL2
RL
siRNA

FIGURE 10

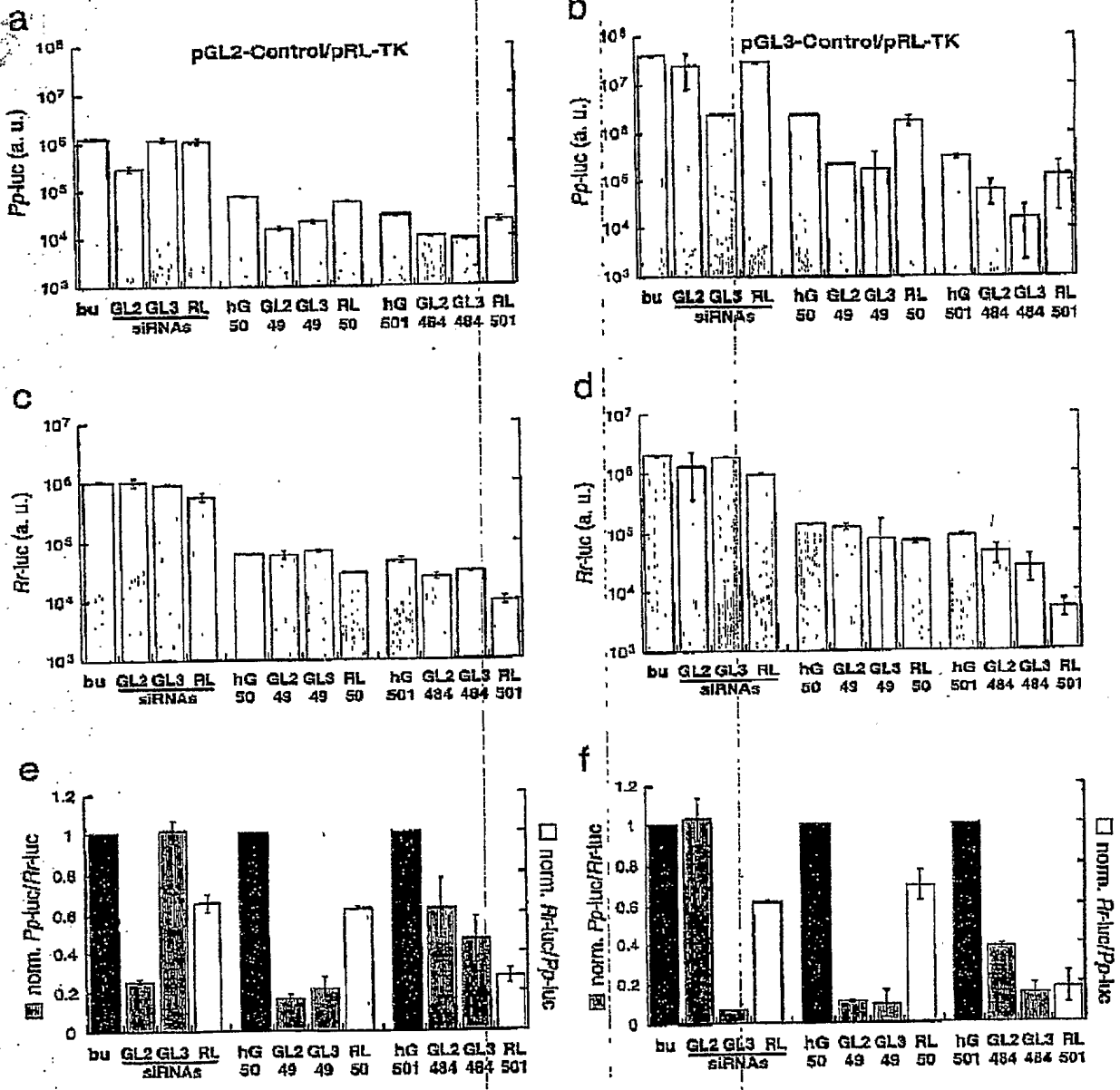

a
pGL2-Control/pRL-TK
Pp-luc (a. u.)
b
pGL3-Control/pRL-TK
Pp-luc (a. u.)
c
Rr-luc (a. u.)
d
Rr-luc (a. u.)
e
norm. Pp-luc/Rr-luc
norm. Rr-luc/Pp-luc
f
norm. Pp-luc/Rr-luc
norm. Rr-luc/Pp-luc
bu GL2 GL3 RL hG GL2 GL3 RL hG GL2 GL3 RL
siRNAs
50 49 49 50 501 484 484 501

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SPECIAL ISSUE

RNA and RNases: Dynamics and catalytic functions of magic molecules

Guest Editors: Gabriele Klug, Reinhard Rauhut and Peter Dürre

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Function, mechanism and regulation of bacterial ribonucleases

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Abstract

The maturation and degradation of RNA molecules are essential features of the mechanism of gene expression, and provide the two main points for post-transcriptional regulation. Cells employ a functionally diverse array of nucleases to carry out RNA maturation and turnover. Viruses also employ cellular ribonucleases, or even use their own in their reproductive cycles. Studies on bacterial ribonucleases, and in particular those from *Escherichia coli*, are providing insight into ribonuclease structure, mechanism, and regulation. Ongoing biochemical and genetic analyses are revealing that many ribonucleases are phylogenetically conserved, and exhibit overlapping functional roles and perhaps common catalytic mechanisms. This article reviews the salient features of bacterial ribonucleases, with a focus on those of *E. coli*, and in particular, ribonuclease III. RNase III participates in a number of RNA maturation and RNA decay pathways, and is regulated by phosphorylation in the T7 phage-infected cell. Plasmid and phage RNAs, in addition to cellular transcripts, are RNase III targets. RNase III orthologues occur in eukaryotic cells, and play key functional roles. As such, RNase III provides an important model with which to understand mechanisms of RNA maturation, RNA decay, and gene regulation. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Bacterial ribonuclease; RNA maturation; RNA decay; Ribonuclease III; Post-transcriptional regulation

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1. Cellular ribonucleases: a riot or regulation?

Many RNA molecules must undergo specific cleavage reactions in order to attain their mature, functional forms. In addition, every RNA ultimately is degraded to mononucleotides. These maturation and decay reactions are carried out by cellular ribonucleases, which exhibit diverse structures and mechanisms of action. Ribonucleases can be grouped broadly into two classes, according to the manner in which they cleave RNA. Endoribonucleases cleave internal phosphodiesters, while exoribonucleases cleave RNA chains from the 3′ or the 5′ terminus, typically in a processive manner, creating mononucleotide products. The end result of cutting and trimming is a properly matured, functional RNA, or mononucleotides which are used in new RNA synthesis.

RNA processing and decay nucleases play important, even essential roles in post-transcriptional regulation. Thus, the rates of maturation and decay, in addition to transcription frequency, establish the steady-state level of an mRNA, and as a consequence determine the rate of synthesis of the encoded protein. Assuming comparable transcription and translation rates, a quickly degraded mRNA will exhibit a lower steady-state concentration and correspondingly lower level of translation than the same mRNA which is degraded more slowly. The employment of unstable mRNAs also allows the cell (or infecting virus) to rapidly shift gene expression patterns, wherein the synthesis of proteins no longer needed can be efficiently stopped through cessation of transcription, coupled with rapid mRNA decay [1].

Many cellular ribonucleases exhibit substantial nonspecific action in vitro. This behavior might be regarded as an intrinsic feature of any degradative nuclease. In fact, RNA processing and decay nucleases are highly selective in vivo, and are responsive to specific signals created by secondary structure and/or sequence elements in RNA. The site of cleavage can determine mRNA half-life, or establish optimal RNA function. Important experimental goals therefore include the determination of the substrate sequence and structural features (reactivity epitopes) that identify ribonuclease cleavage sites, and how ribonuclease activity is regulated. One theme presented in this review is that not only are there RNA signals that promote site-specific cleavage, but RNA signals ('antideterminants') that prevent cleavage. A second theme is that selectivity and control in RNA maturation and decay can be conferred by the association of ribonucleases in multicomponent assemblies. These themes will be discussed within the context of the mechanisms of action of bacterial ribonucleases.

Escherichia coli has been a particularly useful organism with which to characterize cellular ribonucleases and their role in RNA metabolism [2]. A special focus is given to *E. coli* ribonuclease III, which exhibits a number of features that are providing fresh insight into ribonuclease structure and regulation. Eukaryotic ribonucleases will be discussed

within the context of discerned functional and structural similarities to bacterial counterparts. Other reviews should be consulted for comprehensive or focused discussions of bacterial ribonucleases [3–5].

2. Overview of bacterial RNA maturation and decay

The bacterial cell, like all cells, devotes a significant amount of its resources to the synthesis of mRNA and stable RNAs, including tRNA and rRNA. Essentially all stable RNA precursors undergo one or more maturation reactions. For example, bacterial tRNA maturation involves endonucleolytic cleavage by RNase P, which creates the mature 5′ terminus (see also below, and the review on RNase P elsewhere in this volume), while tRNA 3′ end maturation is accomplished through the action of a collection of 3′→5′ exoribonucleases (see also below, and [6]). Ribosomal RNA maturation in bacteria is initiated by endonucleolytic cleavage of the primary transcript during its synthesis. These cleavage events release the precursors to 16S, 23S, and 5S rRNAs, as well as several tRNAs, which are matured by additional endonucleolytic and 3′→5′ exonucleolytic cleavages [7]. The exoribonucleolytic trimming of 3′ ends is a common final step in the maturation of the stable RNAs of *E. coli*, which exhibit a duplex structure created by base-pairing of the 5′ and 3′ terminal sequences [8] (Figure 1).

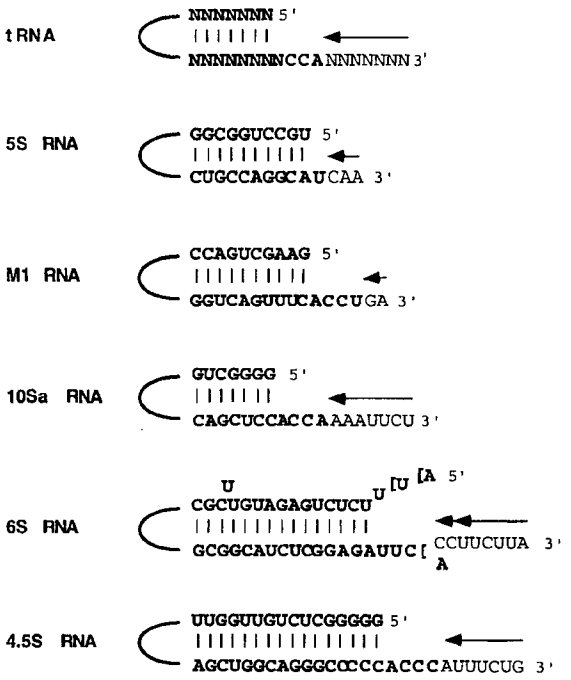


Fig. 1. Processing of the 3′ ends of the small, stable RNAs of *E. coli* [8]. Shown are the proposed base-paired terminal structures for tRNA and five other stable RNA species. The arrows indicate the action of 3′→5′ exoribonucleases (see text). The nucleotides in the mature species are indicated by bold-face.

Bacterial messenger RNA maturation reactions are less common. An important example is provided by T7 phage and its relatives, which synthesize early in infection a polycistronic mRNA precursor that undergoes several site-specific cleavages, providing the mature mono- and dicistronic mRNAs (see also below, and [9]). There also are a number of examples of splicing of bacteriophage mRNAs, which involves endonucleolytic cleavage coupled with ligation (reviewed in [10]).

Bacterial mRNA degradation pathways have now been described. A major pathway for *E. coli* mRNA decay consists of a succession of endonucleolytic cleavages which exhibit an overall 5′→3′ directionality. Closely coupled to these cleavages is the 3′→5′ exonucleolytic digestion of the fragments to mononucleotides [11–13]. Messenger RNA degradation is closely linked to translation, and can be dependent on the frequency of translation initiation. Thus, the initiating step for decay frequently involves site-specific cleavage in the 5′ proximal portion of the mRNA, near the translation initiation site. The 5′→3′ directionality of the subsequent endonucleolytic cleavages may in part reflect the movement of the last translating ribosome [14]. Ribosomes can directly inhibit degradative endonuclease action by occluding cleavage sites [15]. Thus, mRNAs can be stabilized through their active translation.

A major pathway for bacterial mRNA decay involves a multicomponent particle, termed the degradosome, which among other activities contains an endonuclease, RNase E, and an exonuclease, polynucleotide phosphorylase [16,17] (see also below, and the review in this volume). However, mRNA degradation pathways exist which do not appear to involve the degradosome (e.g., see [18,19]). Given the central physiological importance of RNA turnover, it is not surprising that an increasing number of cel-

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lular factors are being identified that are involved in RNA decay and its control. For example, the product of the *E. coli mrsC* allele is involved in normal mRNA decay as well as RNA synthesis [20]. Analysis of the *mrsC* allele reveals it to be the *hflB* gene, which encodes a membrane-associated, ATP-dependent protease [21]. It has been proposed that proteolytic activation of one or more ribonucleases may be important in establishing mRNA decay pathways [21].

3. Bacterial exoribonucleases

There are eight characterized $3' \rightarrow 5'$ exoribonucleases of *E. coli*, which may represent a complete inventory [5,8]. In yeast, a number of $3' \rightarrow 5'$ exoribonucleases participate in a specific assemblage, termed the exosome [22]. However, there is no evidence for a similar association of bacterial $3' \rightarrow 5'$ exoribonucleases. There also has been no report of a $5' \rightarrow 3'$ exoribonuclease activity in *E. coli* or other bacteria. In contrast, eukaryotic cells use at least two $5' \rightarrow 3'$ exoribonucleases as key participants in RNA processing and decay pathways [23].

3.1. Polynucleotide phosphorylase

E. coli polynucleotide phosphorylase (PNPase) is encoded by the *pnp* gene, which maps at 69 minutes on the chromosome [24]. PNPase exhibits an α_3 subunit structure, and uses phosphate as cosubstrate to carry out $3' \rightarrow 5'$ phosphorolysis of RNA chains, producing ribonucleoside 5'-diphosphate products [25]. *E. coli* PNPase is a processive enzyme, but can be blocked by RNA secondary structure. PNPase of *Bacillus subtilis* also is impeded by RNA hairpins [26], and it has been shown to be directly involved in the decay of fragments of the *ermC* mRNA, which encodes a protein conferring resistance to erythromycin [27]. In *B. subtilis*, phosphorolysis rather than hydrolysis may represent the predominant pathway of 3' end-specific RNA decay [28]. *E. coli* PNPase is a component of the degradosome [16,17], and along with RNase II is a primary agent in the exonucleolytic degradation of mRNA fragments (see also below). *E. coli* PNPase also functionally overlaps tRNA nucleotidyl transferase in the repair of the tRNA 3' terminal CCA sequence [29]. *E. coli* PNPase has been shown to bind DNA in a sequence-specific manner [30]. However, the functional importance of this behavior is not known.

PNPase activity is important for cell membrane-associated processes. For example, *B. subtilis* PNPase is required for competence in DNA uptake [31], although the specific functional role has not been determined. *B. subtilis* PNPase$^-$ mutants are sensitive to tetracycline, which may reflect a defect in the export of the antibiotic from the cell [32]. *E. coli* PNPase$^-$ mutants are also sensitive to tetracycline, as well as other antibiotics [33]. It is possible that the PNPase dependence of these cytoplasmic membrane-associated processes reflects the involvement of specific proteins, encoded by mRNAs whose maturation and/or breakdown involves PNPase. PNPase is important for cell growth at low temperatures. Thus, PNPase is a cold shock protein of *E. coli* and of *Photorhabdus* [34,35], and is important for growth of *Yersinia enterocolitica* and *B. subtilis* at low temperatures [32,36]. The importance of PNPase for growth at low temperature may reflect a requirement of ribosome function on a phosphorolytic activity (see below); however, a cell membrane-dependent effect may also be operative.

3.2. Ribonuclease PH

E. coli RNase PH is encoded by the *rph* gene, which maps at 81.7 minutes, and encodes a 25.5-kDa polypeptide. The active form of RNase PH may be an α_2 dimer (reviewed in [37]). RNase PH and PNPase exhibit similar sequences, suggesting a common catalytic mechanism [38]. Indeed, RNase PH also uses phosphate as cosubstrate to degrade RNA, producing ribonucleoside 5'-diphosphates. RNase PH is a conserved activity, as orthologues are present in plants, fungi, worms and mammals [22,38]. A primary role of RNase PH is tRNA 3' end maturation, which also involves the action of several other exoribonucleases [6,37].

RNase PH is not essential for cell viability, which reflects a functional overlap with other exoribonucleases. In fact, an *E. coli* RNase PH$^-$, PNPase$^-$ double mutant is cold-sensitive, which reflects a defect in 50S ribosomal subunit assembly and function [39]. It has not been determined whether the phos-

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phorolytic activity per se, or some other common feature of RNase PH and PNPase is required for large subunit function. This role of RNase PH is apparently conserved, as mutations in a yeast RNase PH orthologue also interfere with large ribosomal subunit assembly and function [40].

3.3. Ribonuclease II

E. coli RNase II is encoded by the *rnb* gene, which maps at 29 minutes [41]. RNase II is a conserved activity, as orthologues have been identified in yeast, mammals, and protozoans, as well as other bacteria [38]. For example, the *Saccharomyces cerevisiae* 3′→5′ exoribonuclease Rrp44p is an RNase II orthologue [22]. RNase II is a Mg^{2+}-dependent phosphohydrolase, and degrades single-stranded RNA, creating ribonucleoside 5′-monophosphate products [42,43]. RNase II exhibits processivity, but can be blocked by RNA secondary structure [43]. Poly(A) is the most reactive substrate for RNase II in vitro [42]. This sequence preference, and the occurrence of 3′ poly(A) tails on mRNA degradation intermediates [11,12], implicates RNase II in mRNA decay. Indeed, RNase II and PNPase are the primary exonucleases involved in mRNA turnover: while the two enzymes can functionally substitute for each other, the loss of both activities is lethal [44].

3.4. Ribonuclease R

E. coli RNase R is encoded by the *rnr* gene, mapping at 95 minutes [45]. The 92-kDa polypeptide exhibits ~60% sequence similarity to RNase II, and the C-terminal domain contains an RNA binding domain similar to that in PNPase [45]. Although RNase R is not essential for cell growth, it shares with PNPase at least one important role, as *E. coli* cells deficient in both RNase R and PNPase are inviable [45]. RNase R is important for expression of the virulence phenotype of *Shigella*, and the enteroinvasiveness of specific *E. coli* strains [45]. The precise involvement of RNase R in establishing these phenotypes is not known.

3.5. RNase D

E. coli RNase D is encoded by the *rnd* gene, mapping at 40 minutes on the *E. coli* chromosome. RNase D is a 43-kDa polypeptide [46,47], and is a divalent metal ion-dependent phosphohydrolase which cleaves RNA chains to produce ribonucleoside 5′-monophosphate products [46,47]. RNase D is involved in tRNA 3′ end formation, although a role in RNA degradation is also possible. RNase D activity may be regulated in the T4-infected cell, through association with a T4-induced polypeptide [48]. Other nucleases containing the conserved RNase D domain are present in fungi, worms and mammals, and include the 3′→5′ exonuclease activity of DNA polymerases [38,49].

3.6. Ribonuclease T

E. coli RNase T is encoded by the *rnt* gene, mapping at 36 minutes, and is a 23.5-kDa polypeptide which is active as an α_2 dimer [50]. RNase T exhibits sequence similarity with *E. coli* DnaQ protein and the family C DNA polymerases [51], and is a member of the 3′→5′ exonuclease superfamily [49]. *E. coli* RNase T participates in the 3′ end maturation of 5S rRNA and tRNAs, as well as the maturation of M1 RNA, 10Sa RNA, 6S RNA, and 4.5S RNA [8]. RNase T is the most effective of the eight exoribonucleases in its ability to remove nucleotides immediately adjacent to the mature, base-paired termini of the stable RNAs (Figure 1), and therefore may provide the final maturation step for these RNAs [8].

3.7. Ribonuclease BN

E. coli RNase BN is encoded by the *rbn* gene, mapping at 88 minutes on the *E. coli* chromosome [52]. RNase BN is a ~60-kDa polypeptide, and participates in the maturation of tRNA 3′ ends [37]. Disruption of the *rbn* gene has no effect on cell growth [52], suggesting a functional overlap of RNase BN with one or more of the other 3′→5′ exoribonucleases.

3.8. Oligoribonuclease

E. coli oligoribonuclease is encoded by the *orn* gene, which maps at 94 minutes [53]. The protein is active as an α_2 dimer, and is highly conserved across phylogeny [53]. Oligoribonuclease is a member of the

3′→5′ exonuclease superfamily [49,53], and therefore probably employs a conserved catalytic mechanism to hydrolyze short oligoribonucleotides to 5′-mononucleotides. A functional role has not been established, but one possibility is that oligoribonuclease carries out the final degradation of short mRNA fragments [53].

4. Bacterial endoribonucleases

The biochemical behaviors and functional roles of five well-characterized *E. coli* endoribonucleases are presented below. There are numerous earlier reports of RNA-cleaving activities in *E. coli* cell-free extracts (reviewed in [4,5]). Some of these activities may be alternate manifestations of characterized endoribonucleases, while others may be distinct, and remain to be fully characterized.

4.1. Ribonuclease I

E. coli RNase I is encoded by the *rna* gene, mapping at 14.3 minutes [54]. RNase I is active as a monomer, with a molecular mass of ~27 kDa. RNase I cleaves within unstructured regions through a substrate-assisted pathway, involving nucleophilic attack of the 2′-hydroxyl group on the adjacent phosphodiester, forming a 2′,3′ cyclic phosphodiester RNA terminus [42]. RNase I is not essential for cell growth, and is present in the periplasm where it may play a role in RNA scavenging. A cytoplasmic form of RNase I, termed RNase I*, has been described [55]. RNase I* activity appears to be masked by a protein inhibitor [56]. The role of RNase I* is unclear, but may be involved in RNA turnover under specific conditions [55,56].

4.2. Ribonuclease III

E. coli RNase III is encoded by the *rnc* gene, which maps at 55 minutes. RNase III is a double-stranded-RNA-specific, Mg^{2+}-dependent nuclease which cleaves phosphodiesters to provide 5′-phosphate, 3′-hydroxyl termini (for reviews see [57–59]). RNase III is active as a 52-kDa homodimer, with a subunit mass of 25.6 kDa (226 amino acids). RNase III participates in the maturation of rRNA precursors, and initiates the decay of specific mRNAs (see also below). Although RNase III is not an essential activity, *E. coli* RNase III$^-$ mutants exhibit slower growth rates, which may be due to a defect in protein synthesis [60]. The decreased translational capacity may reflect a defective 50S subunit containing an incompletely matured 23S rRNA [61].

RNase III is widely if not universally distributed among the eubacteria. The presence of an RNase III gene in the small genome of *Mycoplasma genitalium* [62] underscores the conserved functional importance for a dsRNA-cleaving activity. RNase III of *Rhodobacter capsulatus* (Rc-RNase III) has a high sequence similarity to *E. coli* (Ec) RNase III, exhibits the same divalent metal ion dependence, and can cleave an Ec-RNase III substrate at the canonical site in vitro [63,64]. In contrast, Ec-RNase III cannot cleave an Rc-RNase III substrate, derived from an intervening sequence in *R. capsulatus* 23S rRNA [64]. *Bacillus subtilis* RNase III (Bs-RNase III) exhibits a 36% sequence similarity with Ec-RNase III, and can complement the rRNA processing deficiency of an *E. coli* RNase III$^-$ mutant [65]. A biochemical comparison of Bs-RNase III and Ec-RNase III reveals that Bs-RNase III can cleave an Ec-RNase III substrate at the canonical site, but that Ec-RNase III cannot recognize a Bs-RNase III substrate [66]. The eubacterial RNase III homologues therefore exhibit non-reciprocal substrate specificities, which reflects subtle differences in protein and/or substrate sequence which have not been identified (see also below).

RNase III orthologues have not been detected in any of the sequenced archaeal genomes. In this regard, maturation of the rRNA precursor of the archaeon *Haloferax volcanii* involves cleavage by the bulge-helix-bulge (BHB) endonuclease, which recognizes sites whose positions formally correspond to those of eubacterial RNase III, but which instead exhibit a BHB secondary structure motif [67]. The BHB endonuclease also carries out tRNA intron excision [68,69].

RNase III orthologues occur in eukaryotic cells. The *S. cerevisiae* nuclease Rnt1p exhibits a 20% sequence similarity with *E. coli* RNase III [70], while the *Schizosaccharomyces pombe* Pac1 nuclease exhibits 25% similarity [71]. A comparative biochemical study revealed that Pac1 nuclease cleaves an *E. coli*

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RNase III substrate at the canonical site [72]. However, *E. coli* RNase III fails to recognize a PacI substrate in vitro (I. Calin-Jageman and A.W. Nicholson, unpublished). Interestingly, *S. pombe* and *Caenorhabditis elegans* encode a protein which exhibits an RNase III sequence and a DExH-box RNA helicase sequence [71,73]. The function of this protein in RNA metabolism is not known. Finally, an RNase III orthologue has been detected in the genome of a virus that infects the unicellular alga, *Chlorella* [74]. The function of this protein has not been established.

4.3. Ribonuclease P

E. coli RNase P is a heterodimer of an RNA and a protein. The 377-nt RNA subunit is encoded by the *rnpB* gene at 70 minutes, while the ~13-kDa protein subunit is encoded by the *rnpA* gene at 83 minutes. RNase P is a ribozyme, as the RNA subunit contains the catalytic site, while the protein subunit facilitates substrate recognition (reviewed in [75,76]). RNase P is an essential activity, with both the protein subunit and the RNA subunit required for activity in vivo. RNase P creates the mature tRNA 5′ ends, as well as that of 4.5S RNA, which is a component of the signal recognition particle. A comprehensive discussion of RNase P structure and function is presented elsewhere in this volume.

4.4. Ribonuclease E

E. coli RNase E is encoded by the *rne* gene, which maps at 24 minutes. RNase E is a Mg^{2+}-dependent phosphodiesterase, and cleaves within unstructured, A,U-rich sequences, providing 5′-phosphate, 3′-hydroxyl termini (for reviews, see [12,77], and elsewhere in this volume). Although the predicted mass of the RNase E polypeptide is 116 kDa, the SDS gel electrophoretic mobility indicates a mass of ~180 kDa, suggesting an atypical structure. RNase E is widely distributed among the bacteria [78,79], and functional homologues have been characterized in mammalian cells [80–82]. Although RNase E has been characterized as an endonuclease, a recent report indicates that RNase E can shorten 3′ poly(A) and poly(U) tails in an exonucleolytic fashion in vitro, releasing mononucleotide products [83]. The involvement of this activity in RNA decay has not been established.

RNase E is involved primarily in mRNA decay. However, it has at least one additional role in cleaving the ribosomal RNA transcript, providing the immediate precursor to 5S rRNA [7]. RNase E is an integral component of the degradosome [16,17]. The carboxy-terminal portion of RNase E engages in interactions with PNPase and the RNA helicase, RhlB [78,84,85], while the N-terminal domain contains the catalytic site [86]. A recent study has shown that RNase E action involves recognition of the 5′ end of its substrates. Thus, circularization of an RNase E substrate, or sequestration of the substrate 5′ end-proximal sequence by an antisense oligonucleotide, can inhibit efficient cleavage by RNase E in vitro [87]. Moreover, substrates with monophosphorylated 5′ ends are more reactive than the corresponding 5′-triphosphate-containing RNA [87]. The 5′ end dependence has been proposed to account for the 5′→3′ vectorial behavior of RNase E-dependent mRNA degradation [87]. This behavior would be compatible with the movement of the last translating ribosome [14]. A more extensive discussion of RNase E, and degradosome function in RNA decay pathways is discussed in recent reviews [12,77], and elsewhere in this volume.

4.5. Ribonuclease HI

E. coli RNase HI is encoded by the *rnhA* gene, mapping at 5 minutes on the chromosome. RNase HI is a Mg^{2+}-dependent phosphodiesterase, and cleaves the RNA strand of RNA-DNA hybrids, creating 5′-phosphate, 3′-hydroxyl termini (reviewed in [5,88]). The functional role(s) of RNase HI are obscure, and RNase HI^- mutants are viable. However, the substrate specificity suggests a primary role for RNase HI in DNA metabolism. In this regard, RNase HI plays an essential role in ColE1 plasmid replication, wherein it cleaves an RNA primer to provide a 3′ end that initiates DNA synthesis [88]. A second gene, *rnhB*, encoding RNase HII has been identified, which maps at 4.5 minutes [89]. *E. coli* RNase HII exhibits only a ~17% similarity to RNase HI, and there is no information on its biochemical properties or functional role. RNase HII homologues are widely distributed in the genomes

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Fig. 2. RNase III cleavage of a 13-bp mini-helix substrate. A: The canonical substrate, R1.1[WC] RNA, has been described elsewhere [124]. Mini-R1.1[WC] RNA is derived from R1.1[WC] RNA. The RNase III cleavage sites are indicated by '1' and '2.' The shaded rectangles indicate the proximal box (3 bp) and the distal box (2 bp) (see text). B: Autoradiogram of a cleavage time course assay for the R1.1[WC] RNA and mini-R1.1[WC] RNA. RNAs were enzymatically synthesized in internally ^{32}P-labeled form, and purified by gel electrophoresis. Each RNA (50 nM) was incubated with purified RNase III (10 nM) at 37°C in buffer containing 250 mM potassium glutamate, 10 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.1 mM DTT. Aliquots were removed at the indicated times, and electrophoresed in a 20% polyacrylamide gel containing 7 M urea. The products of cleavage at sites '1' and '2' are indicated. Also, 5′ and 3′ indicate the products containing the 5′ or 3′ terminus of the substrates, respectively. The 5′ cleavage product of mini-R1.1[WC] RNA was present, but too faint to be captured in the image.

of eubacteria, archaea and eukarya [38]. A comparative biochemical analysis of RNase HII of the archaeon *Pyrococcus kodakaraensis* with RNases HI and HII of *E. coli*, indicates that RNases HI and HII are structurally and functionally similar, despite a low sequence similarity [90].

5. Bacteriophage ribonucleases

Bacteriophage T4 encodes two ribonucleases, one of which is involved in T4 DNA metabolism, while the other post-transcriptionally regulates T4 gene expression. The T4 *orfA* (33.2) gene encodes an RNase H activity [91]. The 35-kDa polypeptide is a 5′→3′ exonuclease, and participates in T4 DNA replication by removing the pentameric RNA primers attached to the 5′ ends of newly synthesized chains [91]. In this regard, T4 RNase H resembles the gene 6 exonuclease of phage T7 and the 5′→3′ exonuclease domain of *E. coli* DNA polymerase I [91]. T4 RNase H therefore is mechanistically distinct from RNases HI and HII (see above).

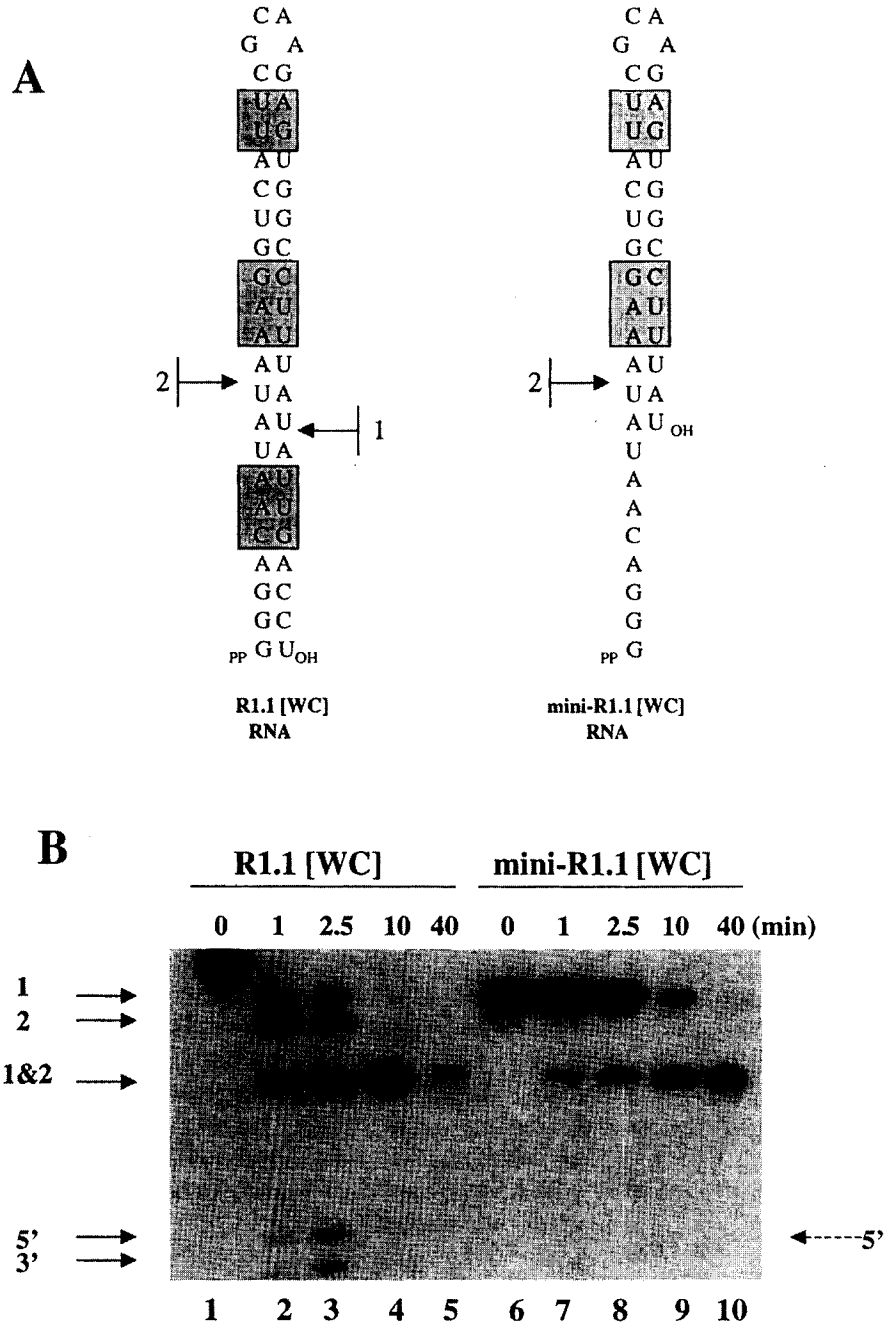
A second ribonuclease activity of T4 is associated with the 18-kDa product of the *regB* gene. The RegB nuclease cleaves within the Shine-Dalgarno sequences of specific T4 mRNAs, down-regulating their translation [92,93]. Since many mRNAs have internal GGAG sequences which are not recognized, additional factors must control RegB nuclease activity. A clue to RegB selectivity is its association with the ribosomal protein S1, which strongly stimulates RegB activity [92]. An in vitro genetic selection analysis which used the RegB-S1 protein combination as the RNA target identified the GGAG tetranucleotide sequence as the only conserved element in the reactive substrates, with an additional preference for flanking A,C-rich sequences [94]. These data indicate that RegB nuclease selectivity for the Shine-Dalgarno sequence reflects a physical association of the nuclease with the 30S subunit [91], which also binds mRNA translation initiation signals. The RegB nuclease provides an important example of the involvement of subcellular localization on control of RNA processing enzyme activity.

6. Function, mechanism and regulation of ribonuclease III

E. coli RNase III plays key roles in both the maturation and the decay of diverse cellular and viral RNAs. These roles, and specific biochemical features of RNase III (see below) underscore the value of this nuclease in understanding ribonuclease function and regulation. This section discusses recent studies on RNase III. Previous reviews [57–59] should be consulted for more comprehensive discussions of earlier research on RNase III.

6.1. RNase III functional roles

A primary role for RNase III is the maturation of the ribosomal RNAs. *E. coli* RNase III cleaves the ~5500-nt primary transcript of the rRNA operons, providing the immediate precursors to the 16S and 23S rRNAs. RNase III recognizes dsRNA structures formed by base-pairing of complementary sequences flanking the 16S and 23S rRNAs (reviewed in [7]). Since these cleavage reactions occur during transcription, the full-length precursor normally is not observed. In several other bacteria, RNase III excises intervening sequences within the rRNA cistrons, creating fragmented yet functional species. For example, RNase III of *R. capsulatus* and of *Salmonella typhimurium* excises intervening sequences in the



23S rRNAs [95,96]. Interestingly, intervening sequence removal is not required for *S. typhimurium* 23S rRNA function, as an RNase III$^-$ mutant retains the intervening sequences, and is viable [97]. It has been proposed that rRNA fragmentation may allow the cell to degrade the rRNA more efficiently during growth under stressful conditions [98].

Eukaryotic RNase III orthologues also participate

in rRNA maturation. The *S. cerevisiae* nuclease Rnt1p site-specifically cleaves the primary transcript within a stem-loop structure in the 3′ external transcribed spacer (3′-ETS), at an early step in the rRNA processing pathway [70,99]. In contrast to the bacterial rRNA precursor (see above), the yeast rRNA precursor is completely synthesized before maturation commences [99,100]. There is evidence that the 3′-ETS not only contains an RNase III cleavage site, but also includes a signal for initiating upstream processing events [99]. The delay in maturation has been proposed as a 'quality control' mechanism, wherein creation of the 3′-ETS indicates correct termination of transcription, thereby signaling that the rRNA sequences have been completely synthesized and are competent for processing [100,101].

Other stable RNAs are created through RNase III action. *E. coli* RNase III cleaves a phage T4 transcript which contains eight tRNAs, and two additional stable RNA species of unknown function (reviewed in [102]). RNase III cleavage is required for proper maturation of the 5′-end-proximal tRNA. Thus, the absence of RNase III causes rapid turnover of the phage tRNA, perhaps due to incorrect folding of the tRNA, which triggers a degradative pathway [103]. *B. subtilis* RNase III cleaves a transcript containing the small cytoplasmic (sc) RNA, which is a component of the signal recognition particle [104]. *S. cerevisiae* Rnt1p cleaves the precursors to the spliceosomal U2 and U5 snRNAs [105,106], as well as a dicistronic transcript encoding the U14 and snR190 snoRNAs [107]. In the latter case, the maturation process is completed by exonucleolytic trimming. It is now apparent that RNase III plays a major role in yeast snoRNA maturation: an *S. cerevisiae* strain which has a disrupted *RNT1* gene exhibits reduced levels of 19 of 42 snoRNAs, with a concomitant accumulation of precursors of these species [108].

As mentioned above, bacterial mRNA maturation reactions are less common, compared to the processing of stable RNAs. However, where such mRNA processing occurs, RNase III is often the participating nuclease. RNase III is solely responsible for maturation of the ~7000-nt polycistronic early mRNA precursor of phage T7, creating the mature mono- and dicistronic species [9]. Several T7 late mRNA precursors are also cleaved by RNase III [9]. Since T7 can grow on *E. coli* RNase III$^-$ strains, these processing reactions are not essential for T7, at least under normal growth conditions, and it remains unclear why the T7 genome is relatively densely populated by RNase III processing signals. This mystery notwithstanding, RNase III action provides stem-loop structures at the 3′ ends of the T7 mRNAs, which contribute to their chemical stability by blocking 3′→5′ exoribonuclease action (e.g., see [109]). T7 mRNAs are not only long-lived, but are also 3′-polyadenylated [110]. These two features suggest the intriguing possibility that T7 mRNAs may be translated as polyadenylated species, and that the poly(A) tail may influence translation, similar to the situation with eukaryotic mRNA [111].

RNase III can initiate mRNA degradation. RNase III cleavage within the 5′ untranslated region (5′-UTR) of its own mRNA promotes subsequent degradation by an RNase E-dependent pathway, which down-regulates RNase III production [112–114]. In the absence of cleavage, the 5′-UTR element is sufficient to stabilize heterologous downstream sequences, while RNase III cleavage destabilizes the transcripts [113,114]. Other examples of RNase III-initiated degradation via 5′-UTR cleavage have been described (reviewed in [115]). RNase III cleavage in the 3′-UTR of the phage lambda integrase mRNA removes a hairpin stem-loop structure, allowing 3′→5′ exoribonuclease degradation of the mRNA and suppressing integrase production [116]. RNase III-dependent RNA degradation can be mediated by antisense RNA. Antisense RNAs recognize complementary sequences in RNA targets, creating dsRNA structures that occlude ribosome binding sites or coding sequences, or which alter target RNA conformation and function [117]. RNase III cleavage of sense-antisense duplex structures irreversibly destroys the ribosome binding sites or coding sequences, thereby enforcing negative regulation [117].

There are several instances where RNase III controls mRNA translation. RNase III cleavage stimulates the translation of at least two T7 mRNAs, apparently by removing structured elements in the 5′-UTR which interfere with 30S ribosome subunit binding (reviewed in [57,58]). The *E. coli* alcohol dehydrogenase mRNA is translationally inactive until site-specifically cleaved in the 5′-UTR by RNase III

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[118]. Cells that lack RNase III fail to grow anaerobically in the presence of glucose, although anaerobic growth can occur with more oxidized carbon sources such as glucuronate [118]. An interesting example of translational control by RNase III processing is provided by plasmid R1, in which cleavage within the coding sequence of a plasmid mRNA directly suppresses production of the protein [119]. Finally, it has been proposed that RNase III can control translation of a phage lambda mRNA in the absence of RNA cleavage [120]. However, there has been no direct biochemical demonstration of such an RNA binding behavior of RNase III.

6.2. RNase III substrate reactivity epitopes

How RNase III selects its cleavage sites has been a perplexing question. For example, although RNase III cleaves cellular and viral substrates in a site-specific manner, RNase III can also degrade dsRNAs of low sequence complexity, providing 12–15-bp duplex products. Moreover, some cellular RNAs exhibit double-helical elements of lengths apparently sufficient for reactivity, yet are not cleaved. Recent studies are beginning to resolve these apparently disparate phenomena, and are revealing that RNase III substrate reactivity epitopes incorporate several structural and sequence features, which are discussed below.

6.2.1. Double-helical structure

RNase III substrates contain a ~15–20-bp dsRNA as a common structural element [57–59,121]. The minimum length that confers reactivity is slightly greater than one α-helical turn. Thus, a 12-bp RNA hairpin, derived from a T7 RNase III substrate and which also contains a single-stranded 5′ extension, can be efficiently cleaved in vitro (Figure 2). However, helix length per se does not select the scissile bond, since either shortening or lengthening the ~22-bp stem of a T7 RNase III substrate does not change the site of cleavage [122]. This contrasts

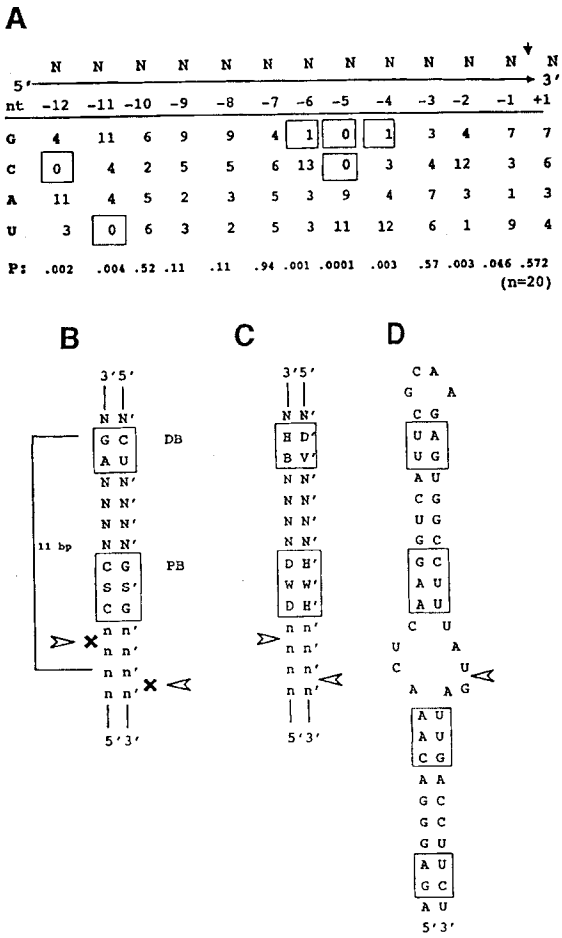


Fig. 3. Position-specific exclusion of W-C bp sequence in *E. coli* RNase III substrates [124]. A: Substrate alignment analysis. Ten substrates were aligned, whose cleavage sites were accurately determined. Each substrate provided two sequences for alignment, reflecting a two-fold symmetry about the RNase III cleavage site (indicated by the vertical arrowhead between nucleotides −1 and +1). The data represent the number of times ($n = 20$) each W-C bp occurs at positions +1 to −12. Position −12 represented the duplex limit for most substrates. P values from a χ^2 analysis are also provided. B: The 'disfavored' W-C bp, displayed in a duplex structure. The rectangles indicate the proximal box (PB) and distal box (DB), which are included within an 11-bp helix. The letter S = C or G, with S′ complementary to S. N, N′ indicate complementary nucleotides, while n, n′ indicate a less strict complementarity. Arrowheads indicate the (blocked) cleavage sites. C: Absence of conservation (degeneracy) of the 'allowable' sequences in the proximal and distal boxes. H = A,G,U, with D′ (A,C,U) complementary to H; B = C,G,U, with V′ (G,C,U) complementary to B; W, W′ = A,U. D: Secondary structure of the T7 R1.1 RNase III substrate [9], showing the proximal and distal boxes, and the single cleavage site within the [4/5] asymmetric internal loop (see also text).

with the yeast tRNA splicing endonuclease, in which the cleavage site is determined by the distance from one end of the dsRNA element [68,69].

6.2.2. Watson-Crick base-pair sequence

The involvement of Watson-Crick (W-C) bp sequence as a reactivity epitope has been controversial [57,58,121,123]. However, it is apparent that RNase III does not behave in the manner of a restriction enzyme, which exhibits a highly specific sequence requirement. To determine whether there is a more subtle involvement of W-C bp sequence, well-characterized RNase III substrates were aligned with respect to their cleavage sites. The alignment revealed that specific W-C bp sequences are underrepresented in two regions relative to the cleavage site: a 3-bp 'proximal box', and a 2-bp 'distal box' (Figure 3) [124]. The W-C bp sequence exclusion is statistically significant, suggesting that the presence of these sequences may influence substrate reactivity. Indeed, incorporation of one or more of the excluded W-C bp in the proximal or distal box positions in a model substrate strongly inhibits cleavage in vitro, due to a weakened binding of RNase III [124].

How might these inhibitory W-C bp act as reactivity epitopes, and select the scissile bond(s)? The W-C bp may 'mask' otherwise reactive phosphodiesters, such that by default, the scissile bond is identified by the absence of the inhibitory W-C bp sequences in the corresponding proximal and distal box positions [124]. Given the constrained set of inhibitory sequences, it follows that the 'allowable' W-C bp in the proximal and distal boxes are degenerate. This sequence degeneracy is in accord with the ability of RNase III to degrade dsRNAs of low sequence complexity, or containing random sequences. In summary, the essential nature of RNase III may be that of largely sequence-nonspecific nuclease, but which carries out specific cleavage of cellular and viral processing signals due to (i) limited helical length, and (ii) a limited set of W-C bp which control cleavage in a position-specific manner.

The inhibitory W-C bp also may serve as protective elements for dsRNA structures with essential intracellular functions. Although it is known that loops and bulges in dsRNA block RNase III cleavage [125,126], these motifs necessarily disrupt regular double-helical structure, which may be required for function. In contrast, the inhibitory W-C bp can block RNase III recognition without disrupting structure. The inhibitory W-C bp, in addition to loops and bulges, can be considered RNase III processing antideterminants. Antideterminants were first described as features of tRNA which assist in the fidelity of tRNA-protein transactions by blocking recognition by noncognate aminoacyl-tRNA synthetases, or translation factors [127]. The realm of action of antideterminants can be broadened to include RNA processing and decay reactions, in which ribonuclease interactions with substrates can be controlled by antideterminant elements. These elements would include 3′ hairpin stem loops, which block $3' \rightarrow 5'$ exoribonucleases [128], and 5′ end hairpin stem loops, which can inhibit RNase E-dependent degradation (e.g., see [129,130]).

6.2.3. Internal loops

A number of RNase III substrates exhibit an internal loop, within which occurs a single cleavage site [9,57–59,121]. Most of the T7 RNase III substrates exhibit an asymmetric [4 nt/5 nt] internal loop, wherein the single cleavage site occurs in the 5-nt strand. Internal loops enforce a pattern of single-strand cleavage. Thus, changing the internal loop of a T7 substrate to fully W-C base-paired form allows coordinate cleavage on both sides of the helix, which is the pattern observed for regular dsRNA [118]. The internal loop also can control the site of cleavage. Thus, a 180° rotational transposition of the internal loop in a T7 substrate equivalently transposes the cleavage site (A.W. Nicholson, unpublished). A functional role for single-strand cleavage is to provide a 3′ hairpin, which can protect the upstream sequence from $3' \rightarrow 5'$ exonucleases.

Comparison of the asymmetric internal loops of T7 RNase III substrates reveals conserved nucleotides, suggesting a specific internal loop structure necessary for site-specific, single-strand cleavage. However, a significant fraction of a T7 RNase III substrate pool which contains a randomized internal loop of 4^9 ($> 200\,000$) different sequences is cleaved at a comparable rate as the single-sequence parent substrate, and at the equivalent site (I. Calin-Jageman and A.W. Nicholson, unpublished). This result argues against a requirement for a specific internal loop sequence (and therefore specific structure) for

reactivity. Nevertheless, specific sequences may confer optimal reactivity: a significant fraction of the T7 substrate pool with a sequence-randomized internal loop is resistant to cleavage (I. Calin-Jageman and A.W. Nicholson, unpublished).

The reactivity epitopes for a consensus *E. coli* RNase III substrate may be summarized as follows. A primary epitope is a dsRNA element of minimal size slightly greater than one helical turn, and which extends in at least one direction from the cleavage site. Many substrates have helical elements extending in both directions from the cleavage site, which may reflect symmetric binding of the homodimeric enzyme to substrate [131]. The cleavage site is determined by the site of RNase III binding, which in turn is dictated by bp sequence. More specifically, the cleavage site is identified by the absence of anti-determinant W-C bp within the 3-bp proximal box and 2-bp distal box. It is possible that the RNase III substrates may tolerate bp mismatches at positions outside the proximal and distal boxes. Finally, an appropriately positioned internal loop can provide the necessary reactivity epitope, directing single-strand cleavage.

Are these *E. coli* RNase III substrate reactivity epitopes transferrable to RNase III orthologues? The core dsRNA element appears to be conserved, and internal loops are occasionally present. However, the inability of *E. coli* RNase III to cleave heterologous substrates (see above) suggests significant differences in reactivity epitopes between RNase III orthologues, perhaps at the level of W-C bp sequence. Also, other motifs may be used by RNase III orthologues: for the *S. cerevisiae* Rnt1p nuclease, a conserved tetraloop hairpin sequence near the cleavage site may be a reactivity epitope [108].

6.3. RNase III structure and mechanism

The dsRNA specificity of RNase III is conferred in part by a specific protein structural feature, occupying the carboxyl terminal one-third of the RNase III polypeptide. The motif is termed the dsRNA binding domain (dsRBD) [132,133], and occurs in many other proteins which bind dsRNA [73,132]. As anticipated, the dsRBD is important for RNase III activity. Thus, a shortened version of RNase III which lacks the dsRBD is defective in vivo (S. Su and R.W. Simons, personal communication) and fails to cleave substrate in vitro in standard assay conditions (E.-J. Jun and A.W. Nicholson, unpublished).

The structure of the RNase III dsRBD has been determined by NMR, and is a compact ellipsoid with an $\alpha\beta\beta\beta\alpha$ tertiary fold [133]. The two α-helices exhibit an approximately parallel orientation on one surface of the antiparallel β-sheet, with the packing stabilized by hydrophobic interactions. The NMR analyses of a dsRBD from *Drosophila* staufen protein, and the tandem dsRBDs in the N-terminal domain of the dsRNA-dependent protein kinase, PKR, reveal essentially the same structures [134,135]. An X-ray diffraction analysis of a cocrystal of a dsRBD from *Xenopus* RNA binding protein A bound to a model dsRNA reveals that the dsRBD interacts with ~16 bp, spanning two minor grooves and the intervening major groove [136]. The significant number of dsRBD-RNA contacts involving 2′-hydroxyl groups on both RNA strands explains the selective recognition of dsRNA, and general insensitivity to specific bp sequence [136].

A mutational analysis of the RNase III dsRBD reveals that specific amino acid changes in the domain inhibit RNase III activity, through interfering with substrate binding. The in vivo severity of the mutations correlates with the loss of dsRNA-binding ability in vitro (A.K. Amarasinghe, A.W. Nicholson, S. Su, and R.W. Simons, in preparation). Residues implicated in dsRNA recognition are clustered at the N-terminus of the α_2 helix, and the connecting loop between β_3 and α_2, although residues important for binding also may be present within the α_1 helix. The RNase III dsRBD in isolated form binds an RNase III substrate with an approximately 100-fold weaker affinity than RNase III. Clearly, the two dsRBDs cooperate in the homodimer to provide the requisite binding affinity, although the remainder of the polypeptide also must engage in substrate contacts (see below). The relatively weak binding affinity of the individual dsRBD may be important for the high catalytic efficiency of RNase III [137], which must incorporate efficient release of the cleaved products in addition to productive binding of substrate. One scenario is that each dsRBD in the RNase III dimer interacts with a helical segment on either side of the cleavage site (e.g., see [131]). Following cleavage,

product release is facilitated by the relatively weak binding affinity of an individual dsRBD to each product helix. Formation of the 5′-monophosphate and 3′-hydroxyl RNA product termini in the active site may provide the signal for product release. The pattern of single-strand cleavage imposed by an internal loop may reflect a disrupted interaction of one of the subunits with a substrate strand.

The RNase III dsRBD is not absolutely required for catalytic activity. Thus, under specific conditions in vitro, a shortened form of RNase III lacking the dsRBD can cleave a small substrate at the canonical site (E.-J. Jun and A.W. Nicholson, in preparation). Thus, site specificity is determined in by dsRBD-independent mechanism, which is consistent with the sequence-nonspecific nature of the dsRBD. Catalytic activity requires one or more protein carboxylate groups. First, treatment of RNase III with a carboxyl-group-specific carbodiimide reagent abolishes catalytic activity without significantly inhibiting substrate binding (G. Xu and A.W. Nicholson, unpublished). Second, sequence alignment of RNase III orthologues reveals eight highly conserved aspartic acid or glutamic acid residues, which are present within the N-terminal domain [71]. Mutation of one of these residues (glutamic acid 117) to either lysine or alanine suppresses cleavage without inhibiting substrate binding [131]. The RNase III [Glu117→Lys] mutant is also catalytically inactive in vivo [138]. It is not known how many divalent metal ions are required for catalysis, nor is it known whether dimerization is required to create the active site(s), or whether each subunit contains its own catalytic site. The preparation of an artificial heterodimer of RNase III, containing a mutant subunit and a normal subunit (e.g., see [139]) may answer this question.

A current picture suggests that the mechanism of RNase III action involves the action of functionally and structurally distinct catalytic and dsRNA binding domains, which synergistically interact to provide catalysis. The dsRBD is required for substrate recognition in vivo, or at physiological salt concentrations in vitro. Since the typical RNase III substrate contains ~2 helical turns, each subunit of the RNase III homodimer may interact with one helical turn in each direction from the cleavage site [131]. The ability of RNase III to cleave a substrate with a ~12-bp stem loop (Figure 2) suggests that the enzyme may only need to employ one subunit for cleavage. This supports the proposal for a catalytic site in each subunit.

6.4. Control of RNase III activity

Regulation of RNase III activity occurs at several levels. First, *E. coli* RNase III regulates its own production by a post-transcriptional mechanism. The mRNA for RNase III is constitutively synthesized, while RNase III cleavage within the 5′-UTR initiates degradation [112–114]. This negative feedback loop establishes a constant level of RNase III. Second, RNase III is subject to covalent control. RNase III is phosphorylated following T7 infection, which stimulates dsRNA-cleaving activity ~4-fold, as measured in vitro [140]. Phosphorylation is dependent upon a T7-encoded, serine/threonine-specific protein kinase, and sites of phosphorylation in vitro include Ser195 and/or Ser198, which are located in dsRBD loop 3 (L.A. Aggison and A.W. Nicholson, unpublished). Phosphorylation also occurs in the N-terminal domain of the RNase III polypeptide; however, the serine target(s) have not been identified.

Why is RNase III phosphorylated in the T7-infected cell? Neither RNase III nor the T7 protein kinase are essential for phage reproduction, at least under normal growth conditions. Nevertheless, T7 synthesizes large amounts of mRNA precursors, using its own highly active RNA polymerase [9]. These precursors are cleaved by RNase III, which in several instances enhances their translation [9,57,58]. Since T7 infection shuts off host transcription [9], phosphorylation would serve to maintain efficient phage mRNA maturation with a fixed amount of RNase III. In this regard it has been shown that phosphorylation of RNase III provides up to an ~8–10-fold enhancement of cleavage in vitro of several T7 RNase III processing signals (L.A. Aggison and A.W. Nicholson, unpublished). Since the T7 protein kinase phosphorylates many other proteins [141], there are additional roles for phosphorylation in the phage reproductive cycle. There is no evidence for a host cell activity that phosphorylates RNase III (L.A. Aggison and A.W. Nicholson, unpublished).

There is some evidence to suggest that RNase III is a ribosome-associated protein. First, RNase III

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cleaves the rRNA precursors, perhaps within the context of partially assembled subunits [7], and a temperature-sensitive mutation in ribosomal protein S12 which affects 30S subunit assembly is suppressed by a mutation in RNase III [142]. Second, RNase III copurifies with ribosomes, and can be released by a salt wash [143]. This behavior is reminiscent of that of translation initiation factors, which associate with the 30S subunit in a salt-sensitive manner. Third, RNase III in cell-free extracts exhibits a sedimentation behavior consistent with its association with a high molecular mass complex [144]. The same study revealed that PNPase exhibits an anomalously high sedimentation rate, which can now be attributed to its association with the degradosome [16,17]. The number of RNase III dimers in the cell is in the order of several hundred, which is substantially less than the number of ribosomes [112]. A reversible, dynamic association of RNase III with the ribosome, coupled with a high catalytic efficiency could allow RNase III to carry out RNA maturation and degradation from a ribosomal locus. A ribosomal site for RNase III would provide a localized high concentration, which would enhance cleavage of the rRNA precursor in partially assembled subunits; and to facilitate cleavage of ribosome-bound mRNAs as part of their maturation or degradation pathways. In this scenario, RNase III would function in a manner analogous to the T4 RegB nuclease, which is selective only for translation initiation regions (see above).

If RNase III associates with the ribosome, what feature(s) of the protein may provide binding affinity? Clues are provided by another dsRNA-binding protein. PKR, which interacts with the small eukaryotic ribosomal subunit via the dsRBD [145]. The ribosomal association of PKR facilitates the phosphorylation of eIF2, which is also a small subunit ligand [145]. It is therefore also possible that the RNase III dsRBD may engage in small subunit contacts. The dsRBD may bind to a specific rRNA segment; however, this mechanism would require RNase III binding without concomitant cleavage. Alternatively, protein-protein interactions could mediate binding.

7. Summary and prospects

There has been rapid progress in determining the mechanisms of bacterial ribonucleases and their roles in cellular and viral RNA metabolism. An important goal will be the completion of the inventory of *E. coli* ribonucleases. A related goal will be to identify the substrate reactivity epitopes for the ribonucleases. These data sets will assist in predicting substrate cleavage sites, RNA maturation and degradation pathways, and perhaps even estimate half-lives for specific RNAs. However, for the last goal, knowledge of additional RNA structural elements and mRNA translational efficiencies will be needed. The archaeal ribonucleases still represent largely unexplored territory. Although the *H. volcanii* BHB endonuclease has been characterized, additional nucleases which hitherto have been resistant to identification through sequence alignments will be identified and characterized by biochemical and genetic approaches.

Determination of the physical structures of ribonucleases will complement biochemical and genetic analyses, and allow the characterization of catalytic mechanisms. As there are only several examples of covalent modification of ribonucleases [146,147], RNase III will provide a useful model for understanding post-translational regulation of ribonuclease activity. Finally, the key involvement of multiprotein assemblies in diverse cellular reactions [148] has been established in RNA metabolic pathways. An ongoing challenge will be to determine the higher-order structures and functions of complex protein (and RNA) machines involved in RNA metabolism.

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